TETX2 – A TETRACYCLINE INACTIVATING ENZYME

TETX2: A TETRACYCLINE INACTIVATING ENZYME

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ABSTRACT

Resistance to the tetracycline antibiotics occurs primarily by efflux and ribosome protection mechanisms, however a tetracycline inactivating enzyme, TetX, was identified 15 years ago, although little is known about this mechanism. The gene encoding this enzyme was identified on a *Bacteroides* transposon and results from DNA sequence analysis and studies of bacterial culture media suggested that *tetX*'s protein product might be a NADPH-requiring oxidase (*Speer BS and Salyers AA. J. Bacteriol. 171(1): 148-153, 1989*). We have expressed a copy of *tetx* gene, *tetx2*, in *Escherichia coli*, and purified the enzyme to high purity. We showed that TetX2 is a monomeric 44 kDa cytoplasmic protein and UV-Vis and HPLC studies established that TetX contained an FAD cofactor. Continuous and stopped enzyme assays have been developed and established that that the enzyme requires O_2 and NADPH for tetracycline degradation. Liquid chromatographic mass spectrometry (LC-MS) analysis of TetX reaction products using oxytetracycline (461 Da) as a substrate indicated that the enzyme catalyses the incorporation of one oxygen atom into oxytetracycline, resulting in a compound of 477 Da with no antibiotic activity. Steady state kinetic analysis demonstrated that TetX2 has a broad substrate specificity with the capacity to inactivate several members of the tetracycline family tested. Identification of the inactivated tetracycline product revealed that the tetracycline inactivation process is a TetX2 catalyzes tetracycline oxidation reaction. These studies provide the first biochemical analysis of a tetracycline inactivating enzyme.

Acknowledgement

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Introduction

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1.1.1 The discovery of tetracyclines

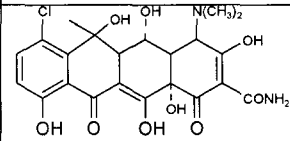
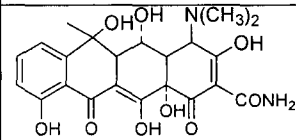
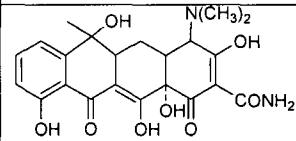
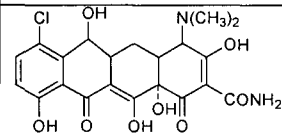
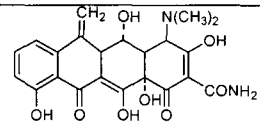
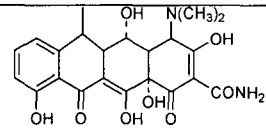
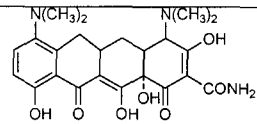
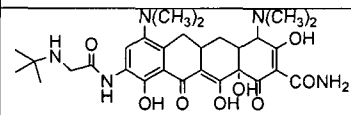
The tetracyclines comprise one of the earliest antibiotics families described and discovered last century. Chlortetracycline was discovered in 1948 (Duggar, 1953) from cultures of *Streptomyces aureofaciens*, this was followed by oxytetracycline, which was discovered from *Streptomyces rimosus* in 1948 (Nelson, 2002), and tetracycline from *Streptomyces viridofaciens* in 1953 (Chopra I., 2001). Other clinically useful members were characterized in the following decades; many of them are semisynthetic compounds, molecules that are synthetic derivatives of natural products (Table 1-1).

1.1.2 Tetracycline biosynthesis

Tetracyclines are a diverse family of compounds produced by the sequential enzymatic synthesis and biotransformation of polyketide precursors, primarily studied within the order *Actinomycetales* (McCormick, et al., 1968 and Hostalek, Z., 1969), other compounds modified with a tetracyclic nucleus can be obtained from these producers, whose member number over 3,000 distinct species in over 40 genera (Nelson, 2002).

Tetracycline biosynthesis begins with the starter unit malonamyl-CoA, which provides the requisite amide at C2. Eight successive condensations with malonyl-CoA, each providing a C2 unit, generate the linear polyketide. This is followed by cyclization to yield 6- methylpretetramid, hydroxylation, oxidation, and reductive transamination

Table 1-1. The principal members of the tetracycline family

Tetracyclines	Year of discovery	Origin	Sturcture of the molecule
Chlortetracycline	1948	*Streptomyces aureofaciens*	
Oxytetracycline	1948	*S. rimosus*	
Tetracycline	1953	*S.viridofaciens*	
Demeclocycline	1957	*S.aureofaciens*	
Methacycline	1965	Semisynthetic	
Doxycycline	1967	Semisynthetic	
Minocycline	1972	Semisynthetic	
Tigecycline	1993	Glycylcycline	

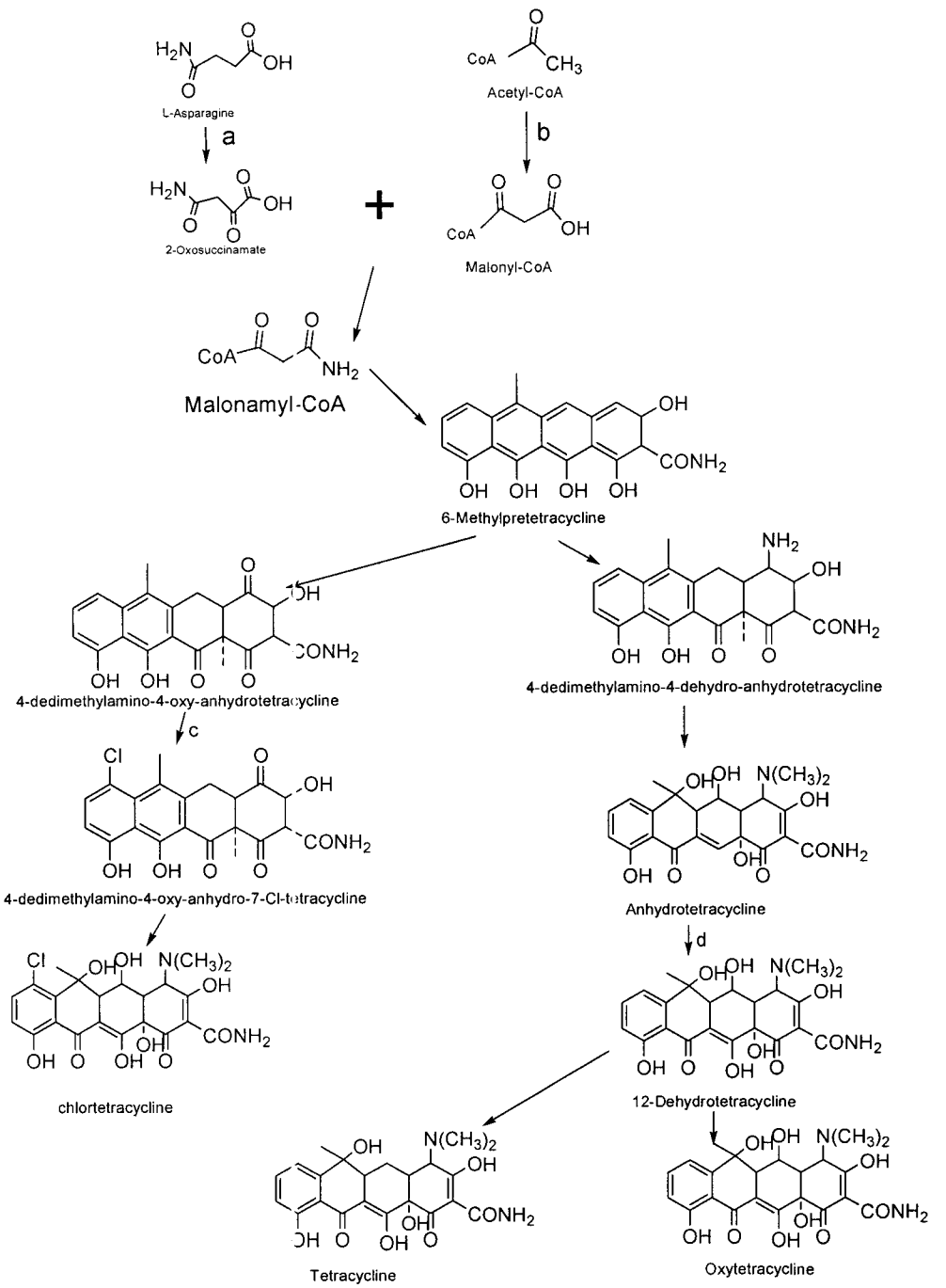


Figure 1-1 Tetracycline biosynthesis pathway. A, b, c, and d are enzymes involved in the biosynthesis. a-Asparagine-oxo-acid transaminase; b-Acetyl-CoA carboxylase; c-tetracycline chlorinbatic n responsible enzyme; d-Anhydrotetracycline monooxygenase.

at C4a as well as hydroxylation at C12a to generate 4-amino-anhydrotetracycline (Wright and Chu, 2003). Addition biochemical reactions result in the biosynthesis of chlortetracycline, tetracycline or oxytetracycline.

1.1.3 Application of tetracyclines

Tetracyclines are broad-spectrum antibiotics with activity against a wide range of Gram-positive and Gram-negative bacteria, mycoplasmas, chlamydiae, mycobacteria, rickettsia, *Helicobacter*, *Listeria* and protozoan parasites such as *Entamodium histolytica, Giardia lambia,* and *Plasmodium falciparum*. Therefore, they have been used extensively to treat human infections since their discovery. For example, community-acquired infections, especially respiratory infections, such as pneumonia caused by *Mycoplasma pneumoniae, Chlamydia pneumoniae,* and *Chlamydia psitttaci*, have been addressed using tetracyclines as the drug of choice for treatment or prophylactic use.

A recent study also reported that a chemically modified tetracycline induced cytotoxic effect against tumor cell line by activating the apoptotic pathway (D' Agostino et al., 2003). Finally, tetracyclines have been used to treat animal infections, plant pathogens, and fish infections (Evens, 2003; Schmidt et al., 2001).

Studies have shown that tetracyclines can enhance the vitamin production by gastrointestinal microorganisms, eliminate the subclinical populations of pathogenic organisms, and increase intestinal absorption of nutrients in animals (Chopra and Roberts, 2001). Thus, oxytetracycline and chlortetracycline have been widely used as animal growth promoters in food production (Sengelov, 2003).

For the treatment of diseases in humans, the semisynthetic compounds doxycycline (Alptekn et al., 2000) and minocycline (Greenstein, 2003; Skidmore et al., 2003) are used more extensively than their natural analogues due to better absorption.

1.1.4 The mode of tetracycline action on bacteria

Tetracyclines are typically bacteriostatic agents that do not kill bacteria but stop bacterial growth by interrupting protein synthesis. Therefore the mode of tetracycline action includes uptake of tetracyclines by bacteria and subsquent binding tetracycline to the ribosome (see section 1.1.4.2 below).

1.1.4.1 Uptake of tetracyclines by bacteria

It has been widely accepted that tetracycline molecules enter susceptible bacteria by passive diffusion through the outer membrane, followed by energy-dependent, pH driven, transport through the cytoplasmic membrane (Schnappinger and Hillen, 1996). A model to explain the uptake process has been discussed in detail elsewhere (Nikaido and Thanassi, 1993). A brief review of this proposal is given below (Fig.1-2).

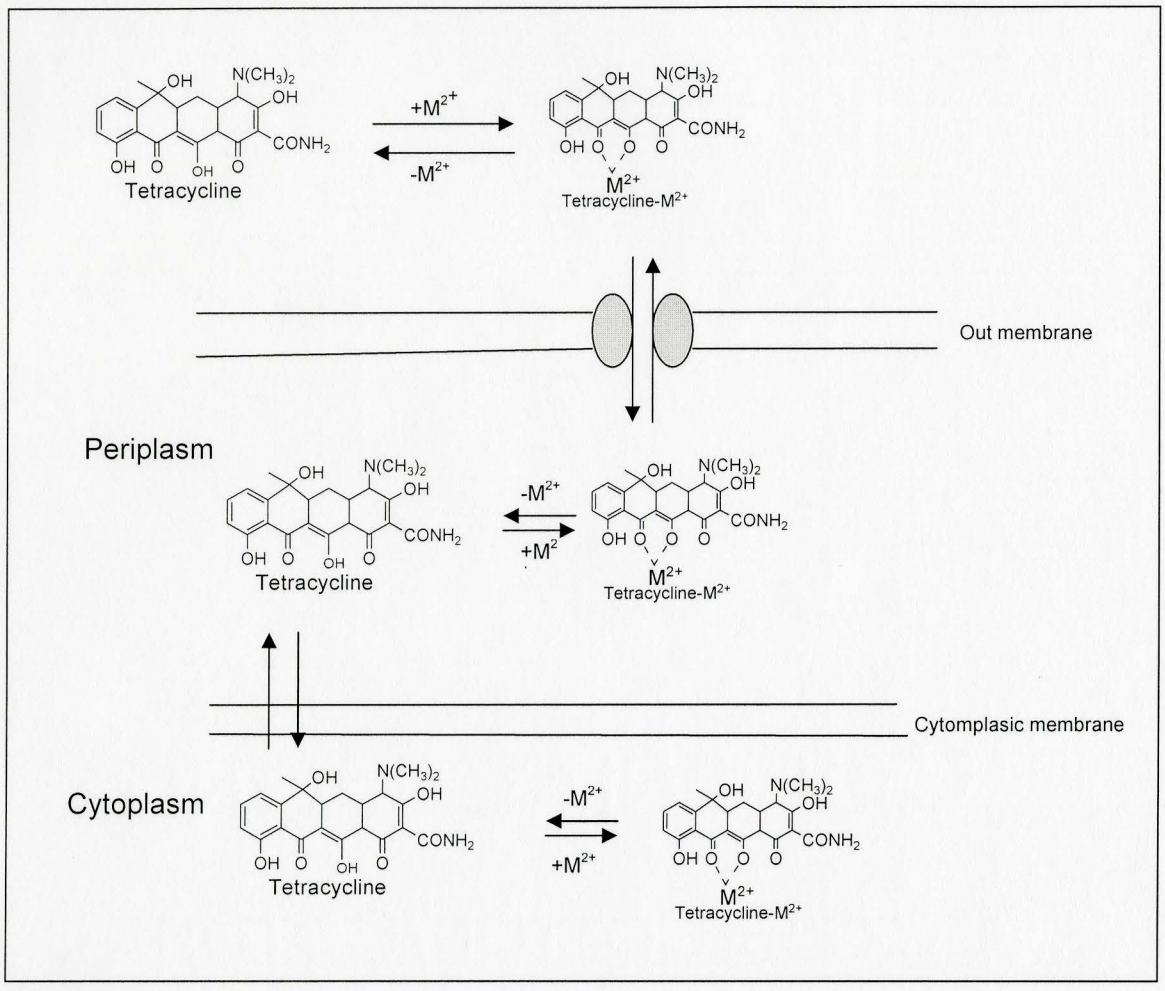


Figure 1-2. Uptake of tetracycline by *Escherichia coli*. M^{2+} represents a divalent metal ion.

A tetracycline molecule crosses the outer membrane of Gram-negative bacteria through the porins OmpF (Thanassi et al., 1995) and OmpC (Mortier and Piddock,1993), probably chelating a divalent cation as a positively charged cation-tetracycline complex and accumulating in the periplasm, then across the by cytoplasmic membrane. It may dissociate and yield into the uncharged tetracycline molecule, a weakly lipophilic compound which is able to diffuse through lipid bilayers (Argast and Beck, 1984). Therefore, tetracycline may penetrate the cell in its electrically neutral form. In the cytoplasm, it may be converted to an ionic compound again since the internal pH and

cation concentration are higher than in the periplasm (Yamaguchi et al., 1991; Nikaido and Thanassi, 1993).

1.1.4.2 Binding of tetracyclines to ribosome

There are three binding sites for tRNA molecules on the small or 30S, ribosomal subunit: the A site for the binding of aminoacyl-tRNA, the P site for the binding of peptidyl-tRNA, and the E site for the exit of elongated peptidyl-tRNA. Primarily, tetracyclines bind to the ribosomal subunit (Ross et al., 1998) where they inhibit protein synthesis by blocking the binding of aminoacyl-tRNA to the A-site. This action is reversible (Roberts, 1996), which may explain the bacteriostatic properties of tetracyclines. The binding is also specific to the ribosome of prokaryotes, which can be used to explain the low toxicity of tetracycline to eukaryotes. Tetracyclines have no apparent effect on the binding of tRNA to the P site (Broderson et al., 2000). They may prevent the binding of both release factors RF-1 and RF-2 during termination, regardless of the presence of the stop codon (Brown et al., 1993).

Early biochemical studies have proposed one primary tetracycline binding site and several secondary binding sites within the ribosomal small subunits (Epe et al., 1987; Kolesnikov et al., 1996). This was supported by the measurement of a 1:1 molar ratio of the tetracycline to ribosome (Goldman et al., 1980) and photoincorporation of tetracycline into *Escherichia coli* ribosomes (Goldman et al., 1983). The important proteins in the 30S subunit for the high affinity binding of tetracyclines include S3, S7, S14, and S19. The 16S rRNA in the region of nucleotides A892 through U1054,

especially U1052 and U1054, may be involved in the tetracycline binding, which were identified by chemical footprinting (Moazed and Noller 1987).

The three dimensional structure of the complex of tetracycline and the 30S ribosomal subunit from *Thermus thermophilus* with a resolution of 3.3 to 3.4 Å has been determined. This localized the tetracyclines binding site on 30s subunit, which agrees well with most biochemical results (Fig 1-3). Two binding sites for tetracycline within the small ribosomal subunit have

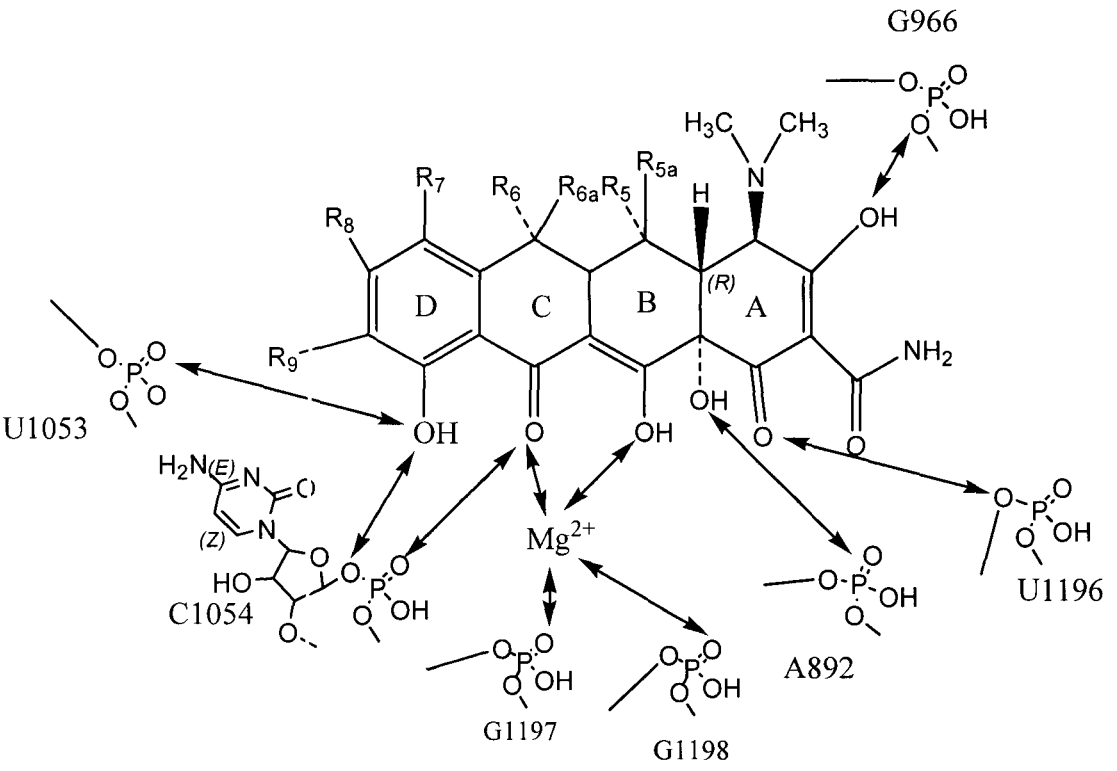


Figure 1-3. The tetracycline binding site on ribosome 30S subunit. Arrows indicate the putative interactions between the ribosome the tetracycline molecule (according to Brodersen et al., 2000).

been found (Broderson et al., 2000). A primary site is located near the A-site and a secondary binding site which is not close to the A site of the subunit. Thus, the mechanism of tetracycline action on bacterial protein synthesis cannot be simply

explained by the disruption of the codon-anticodon interaction between the tRNA and the A-site on the ribosome (Hőgenauer & Turnowsky, 1972) or by the interruption of the interaction of the elongation factor EF-Tu with the A site (Smythies, Benington & Morin, 1972). It is possible that tetracycline does permit presentation of the aminoacyl-tRNA by EF-Tu, which would trigger GTP hydrolysis. Therefore, tetracyclines may act in two ways, first by preventing occupancy of the A-site by the aminoacyl-tRNA and thus arresting translation, and second in a catalytic fashion, depleting GTP stores in the cell (Wright and Chu 2003).

Atypical tetracyclines, such as chelocardin, 6-thiatetracycline, and anhydrotetracycline exhibit antibiotic activity but target the cytoplasmic membrane, not the bacterial ribosome (Chopra 1994). Since their activities are not specific to prokaryotes and have caused serious side effects in eukaryotes, it is not clear whether these compounds have any clinical potential (Roberts, 1996).

1.2 Structure Activity Relationships among Tetracyclines

The study of structure and activity relationships of tetracyclines has been essential in the effort to circumvent bacterial resistance by developing new analogues of the drugs.

Tetracyclines are comprised of a linear fused four carbonic rings (labeled A to D) attached by varies side groups. The molecule is separated into two parts: a hydrophobic region and a hydrophilic region (Fig 1- 4). It has been shown that modification of the groups in the hydrophilic part abolishes the bacteriostatic activity of the compounds (Hlavka and Boothe, 1985; Sum et al., 1998).

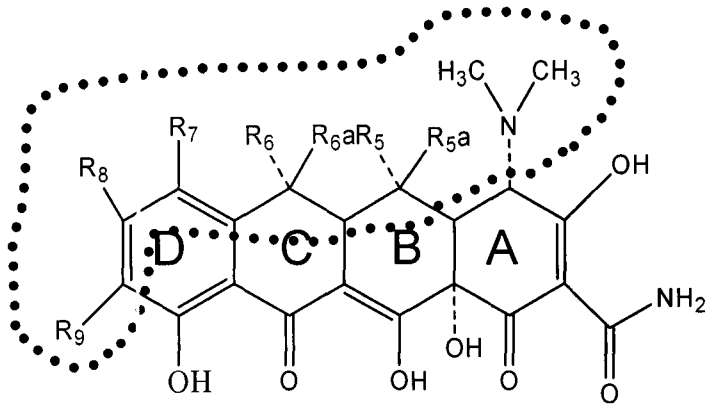


Figure 1- 4. General structure of tetracycline molecules. The circled region is hydrophobic.

On the other hand, variations in the hydrophobic region involving the methyl groups of N4 at ring A and substituents at C5, C6, and C7 to C9 are permissible and provide a high probability of antibacterial activity (Sum et al., 1994; 1999; Chopra and Roberts, 2001). Glycylcyclines are new semisynthetic tetracycline analogues which were created by modifying minocycline at the C9 position, replacing the –H by a modified glycylamido group, now in phase II clinical trails. One of them, tigecycline (GAR 936), exhibits antibiotic activity not just to tetracycline susceptible bacteria, but also to tetracycline resistant strains regardless the resistance mechanism of the strain (Someya et al., 1995; Peterson et al., 1999; Chopra, 2002). This analogue shows some promise for overcoming the problem of tetracycline resistance and is presently in development as a new anti-infective agent.

1.3 Tetracycline resistance

1.3.1 Emergence of the tetracycline resistance

Because of the broad antimicrobial spectrum of tetracyclines, the specificity of activity to prokaryotes, and the low cost of manufacturing, tetracyclines have been widely used to treat infectious and non infectious diseases throughout the world and are the second after penicillin in tons used each year (Roberts 1996; Col & O'Connor, 1992). The consequence of the uncontrolled usage is the emergence of the tetracycline resistance problem. Prior to the mid-1950s, the majority of commensal and pathogenic bacteria were susceptible to tetracyclines (Levy 1984; Chopra et al 2001). The first tetracycline resistant bacterium isolate was *Shigella dysenteriae*. Today, most genera examined have some tetracycline resistant isolates (Roberts, 2003). Even though the usage of tetracycline has been limited, but the spread of existing resistant determinants continues (Takahashi et al, 2002) and new resistant determinants are still being discovered (Melville et al. 2001; Whittle et al. 2003; Torrits et al., 2003). The consequence is the decreased effectiveness of tetracyclines. Therefore, the development of strategies for circumvention of bacterial resistance is one of the most important goals in the treatment of infectious diseases (Schnappinger and Hillen, 1996), as they are still threatening human life. A better understanding of the resistance mechanism will be fundamental to these efforts.

1.3.2 Tetracycline resistance mechanisms

So far, 37 tetracycline resistance genes have been characterized (Table1-3). The designation of the resistance genes used to be determined by DNA-DNA hybridization under relatively high stringent condition (Speer et al, 1991; Chopra and Roberts, 2001). Now it can be performed directly by DNA sequence information. If two genes encode

proteins that have ≤ 79% identical amino acid sequences, they are belonging to different classes; otherwise, they are considered to be closely related (Chopra and Roberts, 2000).

Most of the 37 tetracycline resistant genes encode one of three tetracycline resistant mechanisms: tetracycline efflux, ribosomal protection, and enzymatic inactivation. Mechanisms encoded by *tetU* and *otrC* are undefined or unknown (Table 1-2).

Table 1-2 Characterized tetracycline resistance genes

Genes	Mechanism
tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetI, tetJ, tetZ, tet 30, tet 31, tet 33, tet 34, tet 35, tetK, tetL, tetP(A), tetV, tetY, otrB, tcr3	Tetracycline efflux
tetM, tetO, tetS, tetW, tetQ, tetT, otrA, tetP(B), tet32, tet36	Ribosomal protection
tetx, tet37	Enzymatic inactivation
tetU, otrC	Unknown

1.3.2.1 Tetracycline efflux

Tetracycline efflux is the best studied tetracycline resistance mechanism. There are 22 tetracycline resistance genes that encode tetracycline efflux proteins (Roberts, 2003). These proteins belong to the major facilitator superfamily (MFS) (Bolhuis et al., 1997; Paulsen et al., 1996). The MFS includes over 300 individual proteins. These are membrane-associated, energy-dependent proteins that export tetracyclines from the cell to reduce the intracellular drug concentration and as a result protect the ribosomes within the cell. Each of these proteins is about 46 kDa (Chopra and Roberts, 2001).

The primary amino acid sequences of various efflux proteins have been known for a few years and studies have been conducted to predict the possible secondary structure

of the proteins. A two dimensional topology model originally for TetA(B) has been postulated and used to analyze other tetracycline transporters (Fig 1-5) (Schnapping and Hillen, 1996).

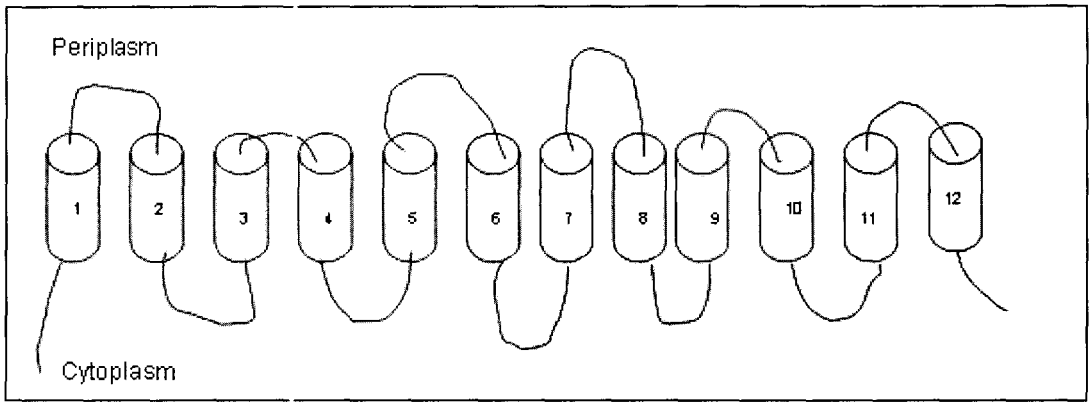


Figure 1-5. The topology of the tetracycline efflux protein of TetA of Tn 10 (Lewis et al., 2003). It shows the 12 transmembrane segments and the 11 connecting loops.

It is suggested that all tetracycline transporters of Gram-negative bacteria share a similar secondary structure (Allard and Bertrand 1993). The model of the Tn10-encoded efflux pump, TetA, comprises two structurally symmetrical halves, each containing six of the 12 transmembrane segments. There are 11 loops connecting the 12 segments (Eckert and Beck 1989a). A series of studies have been conducted to elucidate the function of these loops and specific amino acid residues within them (Schnapping and Hillen 1996; Jin and Krulwich 2002; Lewis et al 2003). Loops protruding into the cytoplasm are between segments 2 and 3, 6 and 7, and 10 and 11. It has been shown that a histidine (His_{257}) in TM8 is essential for its activity (Yamaguchi, 1996; Lewis et al 2003).

Attempts to identify amino acid residues important for tetracycline transporters have concentrated primarily on mutagenesis of charged amino acid and conserved

sequence motifs. A conserved sequence motif GXXXXRXGRR in the cytoplasmic loop connecting segments 2 and 3 has been found (Yamaguchi et al., 1992). This observation suggested that loop 2-3 may be part of the entrance gate of the tetracycline-cation complex. Based on the structural similarities, loop 8-9 might also be located at the entrance of the substrate translocation pathway (Yamaguchi et al 1993; 1998).

Based on amino acid sequence identity, McMurry and Levy (2001) divided tetracycline efflux proteins into six groups (Chopra and Roberts, 2001).

Group 1 contains TetA-E, G, H, Z, and probably TetI, TetJ, Tet30. This group of proteins has between 41 to 78% amino acid sequence identities. All have 12 predicted transmembrane α-helices with long central nonconserved cytoplamic loops connecting helices 6 and 7 (Fig 5). TetZ is the only efflux protein found in Gram-positive bacteria; the others are found only in Gram-negative bacteria (Chopra and Roberts, 2001). Two functional domains, α and β, corresponding to the N- and C- terminal halves of the protein (Rubin and Levy, 1991), are important for the resistance function.

Group 1 efflux proteins are widely distributed and often associated with large plasmids, most of which are conjugative. These plasmids often carry other antibiotic resistance genes, heavy metal resistance genes, and pathogenic factors such as toxins (Chopra and Roberts 2001). This can explain the connection between tetracycline resistance and multi-drug resistance.

TetK and TetL, the group 2 proteins, have 58 to 59% identical amino acid sequences and 14 predicted transmembrane α-helices. They are generally found in small transmissible plasmids, which are easy to integrate into Gram-positive bacterial

chromosome (Su, He, and Clewell, 1992; Gillesspie et al., 1987). Like group 1, they belong to the MFS family.

Group 3 includes OtrB and Tcr3. These genes were found in *Streptomyces* spp. Their topology is similar to that of group 2: with 14 predicted transmembrane α-helices. TetA(P) from *Clostridium* spp., with 12 predicted transmembrane α-helices is in group4. Group 5 includes TetV from *Mycobacterium smegmatis*. Group 6 includes those unnamed *tet* genes (Table 1-2) from *Corynebacterium striatum* and one protein which is believed to use ATP rather than a proton gradient as the energy source (Chopra and Roberts, 2001).

1.3.2.2 Ribosomal protection

Ribosomal protection mechanism of tetracycline resistance was first proposed in 1986 associated with a *tetM* gene in *Streptococcus spp.* was found encode a mechanism different from efflux and enzymatic tetracycline inactivation (Burdentt, 1986). To date, eleven genes encoding ribosomal protection proteins (RPPs, Table 1-3) have been characterized. Among them, TetM and TetO are the most popular ones (Jeric et at., 2002; Ribera et al., 2003; Avial et al 2003). An investigation conducted in the U.K. found that 11% of the total cultivable oral microflora are tetracycline resistant and 79% of them carried *tetM* (Villedieu et al., 2003). However a phylogenetic analysis of RPPs revealed the monophyletic origin of these genes (Aminov et al., 2001), which implied substantial transfer and spreading of resistance genes.

Ribosomal protection proteins are cytoplasmic proteins of about 72 kDa (Speer et al., 1991; Taylor and Chau, 1996). It has been shown that they have sequence similarity

with elongation factors EF-Tu and EF-G (Burdett, 1991). The three dimensional structure at a 16 Å resolution using cryo-EM reconstruction demonstrated that TetO and EF-G have an overall similar shape, and TetO binds to ribosome in a similar fashion as that of elongation factors (Spahn et al., 2001). Furthermore, RPPs exhibit ribosome dependent GTPase activity and require GTP to function (Burdett, 1996). Experiments showed that EF-G and TetM compete for binding on the ribosomes, with TetM having a higher affinity than EF-G. This suggests that these two proteins may have overlapping binding sites and that TetM must be released from the ribosome to allow EF-G to bind (Dantley et al., 1998).

According to a series of studies on the functional complexes of TetO protein by chemical probing, binding assays, and cryo-EM reconstructions experiments (Spahn et al., 2001; Connell et al., 2002), as well as on X-ray crystal structure data (Broderson et al., 2000), Connell and his colleagues proposed a model to elucidate TetO mediated tetracycline resistance (Connell et al., 2003). The model can be simplified as follows: 1.Tetracycline binds to ribosome and cause a comformational change of the decoding site, thus blocks the subsequent aminoacyl-tRNA binding to the A site; 2. TetO binds to the tetracycline blocked ribosome; and 3. triggers the release of the bound tetracycline by changing the conformation of the decoding site; 4. the GTPase activity is activated and is released from the ribosome, leaving the decoding site in a conformation that disfavors tetracycline binding, allowing the aminoacyl-tRNA to compete efficiently for the A-site (Connell et al., 2003). Further studies will be required to validate that this model can represent all the RPPs.

It has been noticed that RPPs do not confer as high a level of tetracycline resistance as that observed for efflux proteins when they were cloned into *E.coli* (Roberts, 1996). However, TetM and TetO widely exist in a number of Gram-negative genera, and clinical isolates harboring these genes have been reported (Ribera et al., 2003).

1.3.2.3 Enzymatic inactivation of tetracycline

Enzymatic inactivation of tetracycline as a resistance mechanism was proposed by Speer and Salyers with the characterization of *tetx* gene (Speer and Salyers, 1989). In 1983, Guiney and colleagues found the clindamycin resistance region of two plasmids pBF4 and pCP1 from *Bacteroides* spp. conferred low level tetracycline resistance when cloned into an aerobically growing *E. coli.* strain (Guiney et al., 1984).

Using the maxicells technique, a cytoplasmic protein of 44 kDa was shown to be the possible product of the tetracycline resistance gene. Studying the expression of the fusion protein of the gene bewteen *lacZ* in strains of *E. coli* indicated that the gene expressed under both aerobic and anaerobic conditions, but did not function if the conditions were anaerobic. Furthermore, it was shown that the inhibition of the function of the gene product under anaerobic conditions was not due to the inhibition of electron transport system but the shortage of oxygen, which implied a requirement for oxygen (Speer and Salyers, 1988).

Experiments using UV-Vis spectrophometry and HPLC studying of the recovered 7-^{3}H labeled tetracycline from the culture media of *tetx*-carrying bacteria showed that the

recovered product was modified by the presence of the *tetx* gene product. Thus the chemical modification of tetracycline was proposed (Speer et al., 1989).

Follow up studies on the action of *tetx* were conducted by adding NADPH or NADH into the cell extracts. It was demonstrated that the *tetx* gene product required NADPH for activity. Sequence analysis revealed that the N-terminal amino acid sequence of the protein had homology with a number of $NAD(P)^+$ requiring enzymes. Thus TetX, the *tetx* gene product, appeared to be an NADP-requiring oxidoreductase (Speer et al., 1991).

Recently, a paper from Salyer's group reported that two copies of *tetx* homologues, *tetx1* and *tetx2,* were neighbored in the *ermF* region of *Bacteroides* transposon CTnDOT (Fig 6). The *tetx2* encodes a predicted protein with 99.0% of the amino acid sequence identical to that of the original gene *tetx*. In contrast, the protein encoded by *tetx1* exhibited only 66% of the amino acid sequence identical to those of *tetx2* or *text* (Whittle et al., 2001).

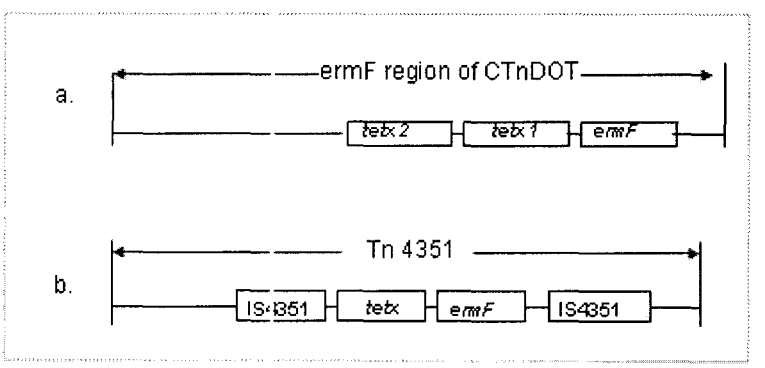


Figure 1-6. Two copies of *tetx* gene are in the *ermF* region of CTnDOT (a) (Whittle et al., 2001), the original *tetx* gene was found on a *Bacteroides* transposon Tn 4351 which also contains a *ermF* gene (b).

Confirmation of TetX is an enzyme that catalyzes the inactivation of tetracycline and how this inactivation process occurs, remains uncertain because of the shortage of biochemical evidence.

Another gene, *tet37,* from the oral metagenome was recently characterized as an enzymatic tetracycline inactivating gene by an unknown mechanism (Torries et al., 2003). However there is no homology reported between the *tet37* and *tetx* gene products.

1.4 Objective of the project

The *tetx* gene was the first gene characterized as tetracycline inactivating determinant. Over a decade later, little further study has been done and no other gene has been characterized in this class. If TetX does encode an enzymatic tetracycline inactivation mechanism, it has to be a catalyst and possess the properties of a catalyst. Therefore, an in vitro approach-including subcloning of the *tetx* gene, overexpression of the TetX protein, and *in vitro* study of TetX protein activity was proposed. Our primary objective was to characterize the enzyme TetX, the requirement for TetX activity, and the product from TetX catalyzed tetracycline inactivation. The gene we used in these studies was the *tetx2* gene described previously (Whittee et al., 2002).

Chapter 2

Materials and Methods

Materials: Tetracycline analogues, $NADP^+$, NADPH, glucose-6-phosphate were purchased from Sigma. Glucose-6-phosphate dehydrogenase came from Roche. Restriction and ligation enzymes were purchased from Fermentas. HEPES, MES, TAPS, CHES were obtained from Bioshop. Trifluoroacetic acid (TFA), acetonitrile, and ammonium acetate were from Caledon.

2.1 PCR

PCR was carried out on a Progeny 96 well themocycler with a program of 95 °C for 1 minute, 52 °C 1 minute, 72 °C for 1.5 minutes, which was repeated for 30 cycles. The template used was genomic DNA of *Bacteroides* transposon BT5482 A Ω CTnDOT, a gift from Dr. Salyers at the University of Illinois. Primers were *tetx2*F: 5'-CCG GAA TTC CAT ATG ACA ATG CGA ATA GAT ACA GAC; *tetx*2R: 5'- CCG GAA TTC AAG CTT TTA T TA TAC ATT TAA CAA TTG C. Restriction sites are underlined.

2.2 Subcloning

A 1.2 kb PCR product was excised from a 0.8% agarose gel, extracted by the QIAquick Gel extraction kit, digested with *Nde*I and *Hind* III, and ligated into pET28 (Novagen), which was digested with the same restriction enzymes. This generated a

fusion construct that gives an N-terminal Hexa-His tagged protein for ease of purification.

Recombinant plasmid with *tetx2* was transformed into *Escherichia coli* strain BL21 (DE3) and transformants were selected by kanamycin (50 μg/ml) resistance. The correct gene sequence was verified by sequencing at MOBIX Central Facility of McMaster University.

2.3 Protein expression

A single colony of *E. coli* BL21 (DE3) contained pET28-*tetx2* was inoculated into 2 ml of Luria Broth plus 50 μg/ml kanamycin and incubated at 37 °C, 250 rpm, for 8 hours. A 250 μl aliquot of the culture was inoculated into 25 ml of LB plus kanamycin and grown at 37 °C, 250 rpm for 12-16 hours. A 10 ml aliquot of the culture was inoculated into 1 litter of LB plus kanamycin, grown at 37 °C, 250 rpm to an OD_{600nm} of approximately 0.6, then isopropyl-beta-D-thiogaltopyranoside (IPTG) was added to a final concentration of 1 mM to induce the expression of TetX2. The culture was incubated at 16 °C with shaking overnight. Cells were collected by centrifugation at 8000 rpm for 5 minutes. Cells were then suspended in 10 ml of 20 mM HEPES buffer (pH 8.0), 1 mM PMSF and 5μl of DNase were added to the cell suspension. The cell suspension was lysed by three passes through a French press at a maximum pressure of 10,000 psi. The cell lysate was clarified by centrifugation at 15,000 rpm for 15 minutes.

2.4 Protein purification by Ni-affinity and Ion-exchange chromatography

The supernatant was applied to a 1 ml Ni-agarose column (QIAGEN) equilibrated with 20 mM HEPES (pH 8.0) and the protein was purified by Fast Performance Liquid Chromatography (FPLC). A linear gradient from 100 % buffer A (20 mM HEPES, pH 8.0) to 100 % buffer B (20 mM HEPES, 250 mM imidazole, pH 8.0) with a flow rate of 1 ml/minute was used to elute TetX2. Selected fractions were analyzed via electrophoresis through an 11% Sodium Dodecyl Sulfate PolyAcrylamide Gel to determine the size and purity of the protein. If necessary, an additional chromatographic step over a Mono-Q (HR 5/5) column (Pharmacia Biotech) was used to eliminate impurities that came along with TetX2 from Ni-affinity column. This column was equilibrated in 20 mM Tris (pH 7.5), and TetX2 was eluted with a linear gradient in 20 mM Tris (pH 7.5) + 1M NaCl over 20 column volumes.

2.5 Gel filtration chromatography

To estimate the size and molecular weight of TetX2, gel filtration chromatography was performed by using a Superdex^{TM} 200 HR10/30 column. Protein standards and sample were prepared, and eluted by following the manufacture's instruction (Sigma MW-GF-200). The UV absorbance at 280 nm was visualized to determine the appearance of the proteins.

2.6 Assays for enzyme activity

2.6.1. Reaction mixture: A typical 100 µl reaction mixture consisted of 1 mM $NADP^+$, 40 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase (0.2 U). These were incubated at room temperature for 5-10 minutes to generate NADPH. This

was followed by adding of 25 mM TAPS (pH 8.5), 0.3 mM tetracycline substrate, and 10 μg of TetX2. The reaction was incubated at 30 °C.

2.6.2. Microtitre plate reader: A Molecular Devices SpectraMax Plus microtitre plate reader (96 wells) was used to record the progress curves of the continuous reaction during a 15 minutes incubation period with the above reaction mixture. The change in the absorbance of oxytetracycline at 400 nm (ε_{400}= 1080 $M^{-1}cm^{-1}$) was monitored.

2.6.3. HPLC: The enzyme activity of TetX2 was also determined by a discontinuous assay. Seven reactions were set up as described in step 1 and were stopped by adding 1% trifluoroacetic acid (TFA) after certain amount of incubation time (0 minute to 30 minutes). An 85 μl aliquot of the 100 μl mixture was taken from each of the stopped reaction mixtures and applied to an Alltech Econosil C18 column (10U, 250 x 22 mm). A linear gradient from H_2O + 0.05% TFA to 75% acetonitrile + 0.05% TFA in 30 minutes with a flow rate of 1 ml/minute was used to separate the substrate and the reaction product(s). Chromatograms at 260 nm and 363 nm were recorded.

2.6.4 Microbiological assay: A 15 μl aliquot of a 100 μl reaction mixture was applied on a piece of Schleicher & Schuell Filter paper (1/4") and allowed to air dry for 45-60 minutes. *Micrococcus luteus* of an OD_{625nm} value of 0.008 to 0.01 was evenly spread on a Tryptic Soy agar plate. The discs with samples were then placed on the top of the agar and incubated at 30 °C for 48 hours.

2.6.5 Spectrophotometry: Reactions were set up as described in 2.6.1. The reaction volume was scaled up to match the volume of the cuvette. For monitoring the difference spectra, there was no TetX2 in the reference cuvette, therefore, no reaction would occur. TetX2 was added into the sample cuvette to start the reaction. The difference of the absorbances of oxytetracycline from 220 to 450 nm in two cuvettes was shown as difference spectrum and recorded every 20 second over a 20 minutes period. To monitor the direct changes in the absorbance spectrum of oxytetracycline during the reaction, oxytetracycline was omitted from the reference cuvette.

2.7 Flavin detection

2.7.1 Flavin detection by spectrophotometer: To detect the bound flavin of TetX2, 200 μg of the protein was boiled for 5 minutes and centrifuged briefly. The absorbance spectrum of the sample from 220 nm to 500 nm was then monitored.

2.7.2 Flavin detection by HPLC: To identify the type of flavin in Tetx2, the boiled protein was applied to a C18 column (Alltech Econosil, 10U, 250 x 22 mm) equilibrated with 5 mM ammonium acetate (pH 6.0) and the bound flavin was separated by a linear gradient of 5 mM ammonium acetate (pH 6.0) to 100% methanol in 20 minutes with a flow rate of 1ml/ minute. Standard FMN, FAD, and riboflavin samples were also run.

2.8 Steady-state kinetic studies

Initial rate studies consisted of duplicate reactions with different concentration of tetracycline or NADPH as requiring. Steady state kinetic parameters were determined by

fitting the initial rate (v) to the standard Michaelis-Menten equation using the Grafit 4 software (Erithacus software limited):

$$(1) \qquad v = k_{cat}[E_0][S]/([S]+K_m)$$

where E_0 is the total enzyme concentration.

2.9 MICs

The antibitotic minimal inhibition concentrations were determined by broth dilution in Luria Broth medium. *E.coli* BL21 (DE3) with pET28 and *E.coli* BL21 (DE3) with pBR322 were used as negative and positive controls respectively.

2.10 NMR spectrum of inactivation product P1

Four 20 ml reactions each consisting of 5 mg of oxytetracycline (3 mM final concentration), 1 mM of NADP, 40 mM of glucose-6-phosphate, 40 units of glucose-6-phosphate dehydrogenase, 25 mM of TAPS (pH 8.5), 1 mg of TetX2 were set up and incubated at 30 °C for 20 minutes. After analytical HPLC analysis, the reactions were freeze-dried overnight until the volume was decreased to less than 5 ml. The residue was then applied to water equilibrated C-18 Sep-Pak mini column (previously washed with methanol). Oxytetracycline inactivation product P1 was eluted with a wash of 30 ml of 1 % acetonitrole in water while collecting 2 ml fractions. Each fraction was analyzed by UV-Vis spectrum and analytic HPLC. Fractions with pure P1 were pooled and lyophilized. The dry product was dissolved in methanol plus 3 drops of D_2O and its ^1H and ^{13}C NMR spectra were determined on a Bruker 600 mHz NMR instrument by Dr. Donald Hughes, Department of Chemistry, McMaster University.

2.11 Liquid chromatography with mass spectrometry detection (LC-MS)

A 500 µl of reaction mixture including the components as described in 2.6.1, but 1 U of glucose-6-dehydrogenase and 50 µg of TetX2 was incubated at 30 °C for 15 minutes and stopped by adding of 1 % TFA. The reaction was then analyzed by LC-MS using a C18 column and a Waters Micromass Quattro Utima instrument in the Department of Chemistry, McMaster University, by Dr. Kirk Green.

Chater 3

Results

3.1 Amplification of *tetx2*

Using the genomic DNA of *Bacteroides* CTnDOT BT5482A Ω, and primer pairs complementary to the 5' terminal and 3' terminal of the *tetx2* gene (Genbank accession number: #CAC47932.1), a 1.2 kb DNA fragment was amplified by PCR (Fig3-1). This band is consistent with the predicted size of the *tetx2* gene in Genbank. The correct DNA sequence was verified by sequencing of the entire *tetx2* gene at MOBIX Central Facility of McMaster University.

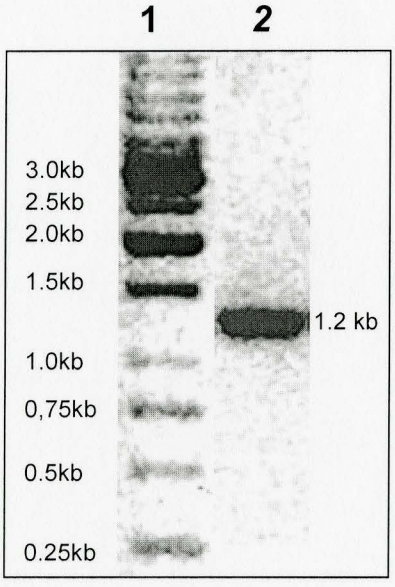


Figure 3-1. PCR amplification of *tetx2* gene. The 1% agarose gel was visualized by Ethidium bromide staining. Lane 1: standard molecule size markers; Lane 2: PCR product. Intervening lanes have been digitally removed for clarity.

3.2 Subcloning and expression of hexa-His TetX2

The *tetx2* gene was inserted into plasmid pET28 (from Novagen) and the construct was used to transform *E. coli* strain BL21 (DE3). This fuses the *tetx2* gene to an N-terminal hexa-His tag and places the gene under control of a T7 promoter.

The hexa-His tagged TetX2 protein was overexpressed and purified by Ni-affinity column. A 44 kDa band was observed on an 11% SDS-PAGE gel consistent with the predicted molecular weight of hexa-His TetX2 (44.4 kDa) (Fig. 3-2a). For simplicity, hexa-His TetX2 will be written as TetX2 in this thesis.

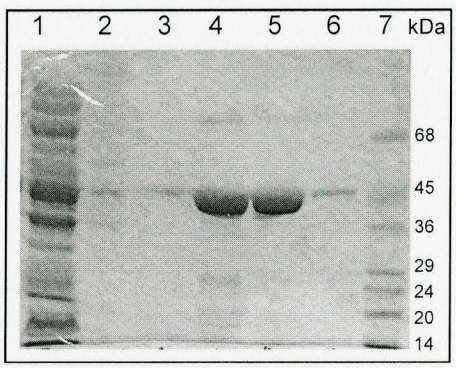


Figure 3-2a. Ni-agarose column purified hexa-His TetX2. The 11% SDS-PAGE gel was visualized by Commassie blue staining. Lane 1: Total soluble protein; Lane 2: High molecular weight marker; Lane 3 to lane 6: Eluted fractions with TetX2; Lane 7: Lower molecular weight marker.

To verify the effect of different reading frames (fig.3-2c) on TetX2 activity, different versions of TetX2 were expressed and purified by Ni-affinity column (Fig 3-2b). Their tetracycline inactivation activities were demonstrated by kinetic data (Table 3-1).

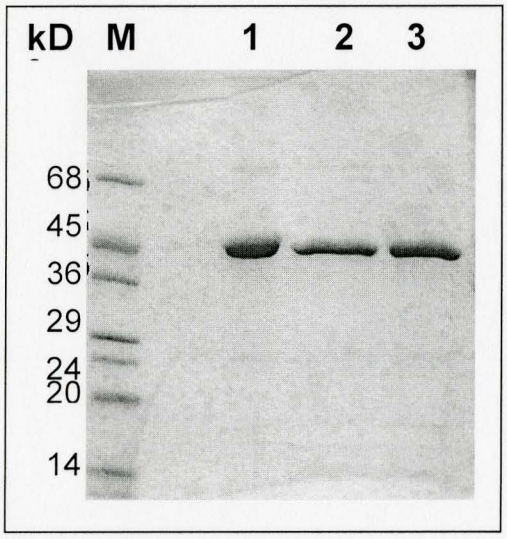


Figure 3-2b. Different versions of TetX2 purified by Ni-affinity column. Bands were visualized by Commassie blue staining. M-molecular weight marker; 1- TetX2.1 starts from the 1st start codon; 2- TetX2.3 starts at the 3rd start codon; 3- TetX3 starts at the 3rd start codon with a mutation of $E_{266}K$.

Position	1	3	11	94	266	360	389	390
TetX	M	M	M	K	E	M		V
TetX2.1	M	M	M	E	E	I	N	
TetX2.3			M	E	E	I	N	
TetX3			M	K	K	M		V

Figure 3-2c Sequence comparison of different version of TetX. TetX: the original version from Tn 4351; Tetx2.1: TetX2 starts from the 1st start codon; TetX2.3: TetX2 starts from the 3rd Start codon; TetX3: A mutant version of TetX start from the 3rd start codon with a mutation of E266K.

Table 3-1 Comparison of the tetracycline inactivation activities of different versions of TetXs

TetXs	Km (μM)	k_{cat} (s^{-1})	k_{cat}/km ($M^{-1}s^{-1}$)
TetX2.1	129 ± 24.3	1.2 ± 0.07	8.45×10^3
TetX2.3	76.3± 16.3	1.3 ± 0.09	1.74×10^4
TetX3	117 ± 26.5	1.0 ± 0.09	8.94×10^3

Oxytetracycline were used as the substrate, 1 mM NADPH, 25 mM TAPS, pH8.5, 10 μg each of the TetXs were used.

Gel filtration analysis over a Superdex^{TM} 200 column revealed that the molecular weight of hexa-His tagged TetX2 is 46 kDa (Fig. 3-3), which is consistent with a monomeric protein.

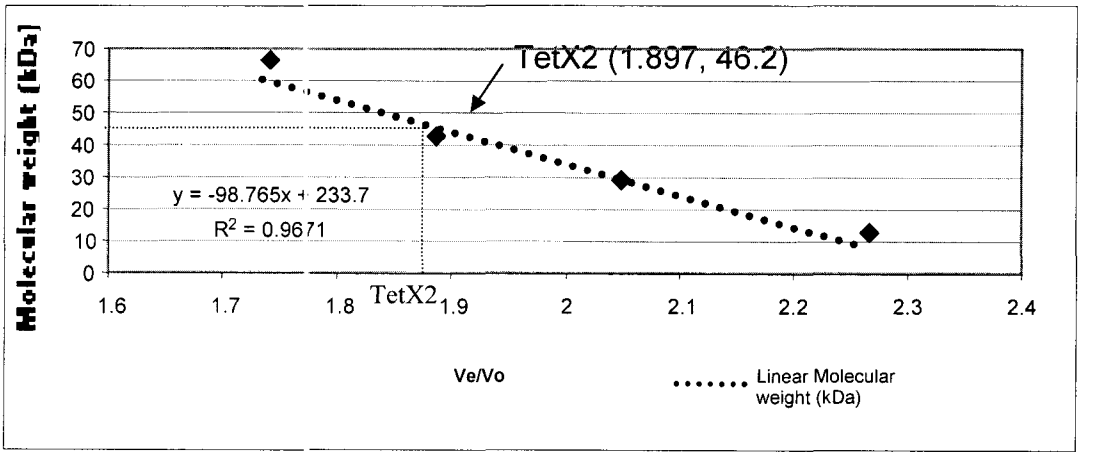


Figure 3-3. Analytical gel filtration of purified hexa-His TetX2. The standard proteins are cytochrome C (12.4 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (43.0 kDa), serum albumin (66.0 kDa).

3.3 TetX2 is a flavoprotein

Purified TetX2 has a bright yellow color. Its UV-Vis. spectrum showed two maxima at 366 nm and 445 nm, which are consistent with a flavin cofactor (Fig 3-4). HPLC analysis of the flavin cofactor isolated from denatured TetX2 was identified as FAD (Fig. 3-5).

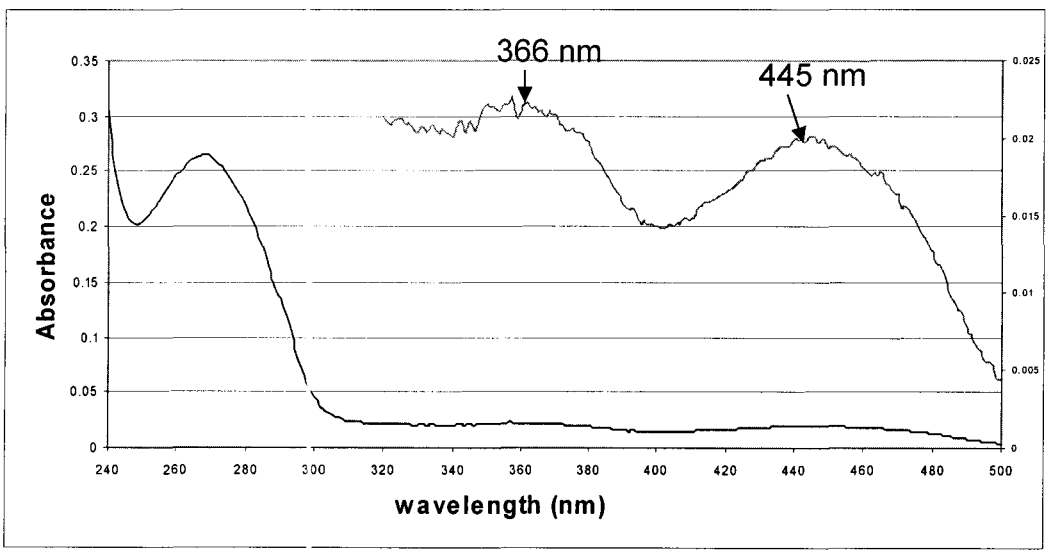


Figure 3-4. UV-Vis spectrum of TetX2. The expansion of 300 – 500 nm region shows the two maxima absorbance peaks of flavin.

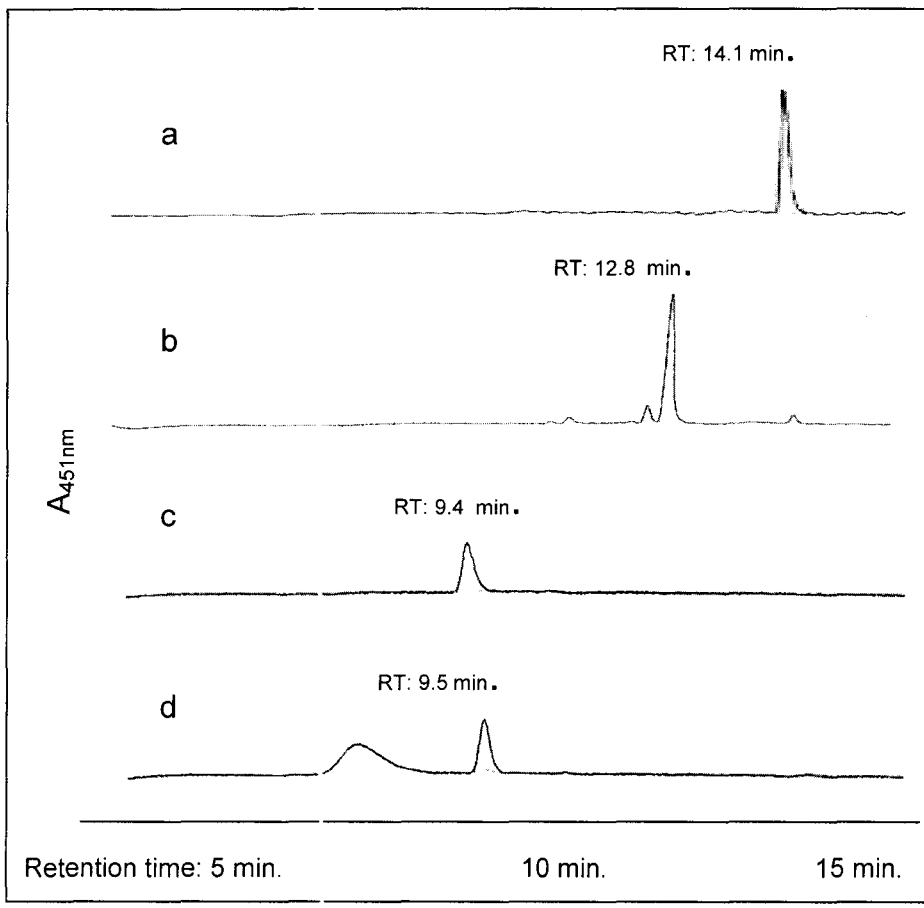


Figure 3-5. HPLC analysis of flavin content in hexa-HisTetX2. Samples: a-riboflavin; b-FMN; c-FAD; d-TetX2. The peaks were detected at 451 nm. RT: retention time. The flavin peak from denatured TetX2 eluted at a retention time that consistent with FAD.

3.4 Optimized conditions for storing TetX2

It was noticed that TetX2 would lose its tetracycline inactivating activity if it was stored at 4 °C for more that 3 days. Therefore, different stabilizing agents including glycerol, 1 mM glutamine, 10 μM FAD, and 30% ammonium sulphate were examined as optimal storage agents. Purified TetX2 (1.5-3.0 μg/μl) was stored at -80 °C with the addition of the above materials. After 3 or 5 days of storage, the oxytetracycline inactivation rates were measured (Table 3-2). Comparing TetX2 without the addition of other chemicals, the addition of 10 μM of FAD can keep the enzyme activity the same as that of the fresh one (Vo =1. 2 nmole/minute) or slightly higher. The latter can be explained as by the loss of FAD during purification of the fresh protein.

Table3-2 The effect of potential stabilizing agents on the activity of TetX2 during storage

Storage at -80 C (days)	*TetX2*	*+5%Glycerol*	*+10%Glycerol*	*+1mM Glutamine*	*+10μM FAD*	*+30% Ammonia Sulphate*
3	1.14*	1.04	0.98	1.07	1.47	0.67
5	1.07	0.92	0.90	1.01	1.41	0.64
Difference	6.1	11.5	8.2	5.6	4.1	4.5

* Numbers are the reaction rates (Vo: nmole/minute). Difference was obtained by using formula (day 3-day 5)/ day 3 x 100 %.

3.5 Optimal pH and buffer for TetX2

The pH profile for optimal activity (Fig 3-6) was determined by using MES (pH 5.5 – 6.5), HEPES (pH 6.5 – 7.5), TAPS (pH 7.5-8.5), CHES (pH 8.5 – 10) as reaction buffers. The decrease of the absorbance at 400 nm of oxytetracycline was measured using a microtitre plate reader over 15 minutes. The reaction rate in Fig. 3-6 is the initial reaction rate of TetX2 catalyzed tetracycline inactivation reaction. The highest rate was

obtained when the TAPS buffer was used at pH 8.5 (Fig.3-6). Therefore, the TAPS buffer of pH 8.5 was selected as the optimal buffer condition for TetX2, and it was used for all of the following assays in this project.

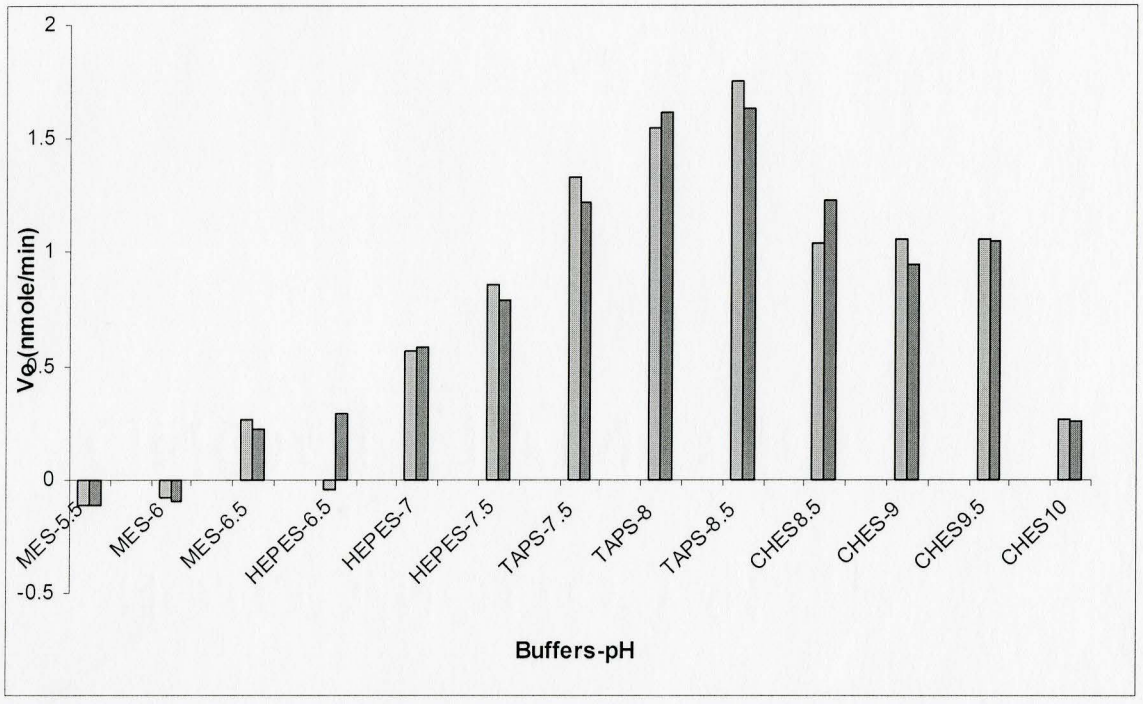


Figure 3-6. Optimal buffer for TetX2 function. X-axis are different buffers; Y-axis is the reaction rate, Vo. Results from duplicate assays were shown by neighbored bars. TAPS buffer at pH 8.5 gave the highest rate.

3.6 The effect of co-factors on the activity of TetX2

Primary sequence analysis predicted that TetX might be a $NADP^+$ requiring oxidoreductase (A. Speer and S. Salyer, 1991). To test this assumption *in vitro*, different cofactors were used and the enzyme activity was determined (Fig. 3-7). Tetracycline is known to bind divalent cations, thus Mg^{2+} was also investigated. The presence of NADPH gave the best result, followed by the NADPH regenerate system ($NADP^+$ +

glucose-6-phosphate + glucose-6-phosphate dehydrogenase) gave the second. Mg^{2+} did not increase the reaction rate, but did slow it down slightly. The presence of NADH had almost no effect compared with the control (no cofactor used). Therefore, NADPH is required exclusively for TetX2's tetracycline inactivation activity.

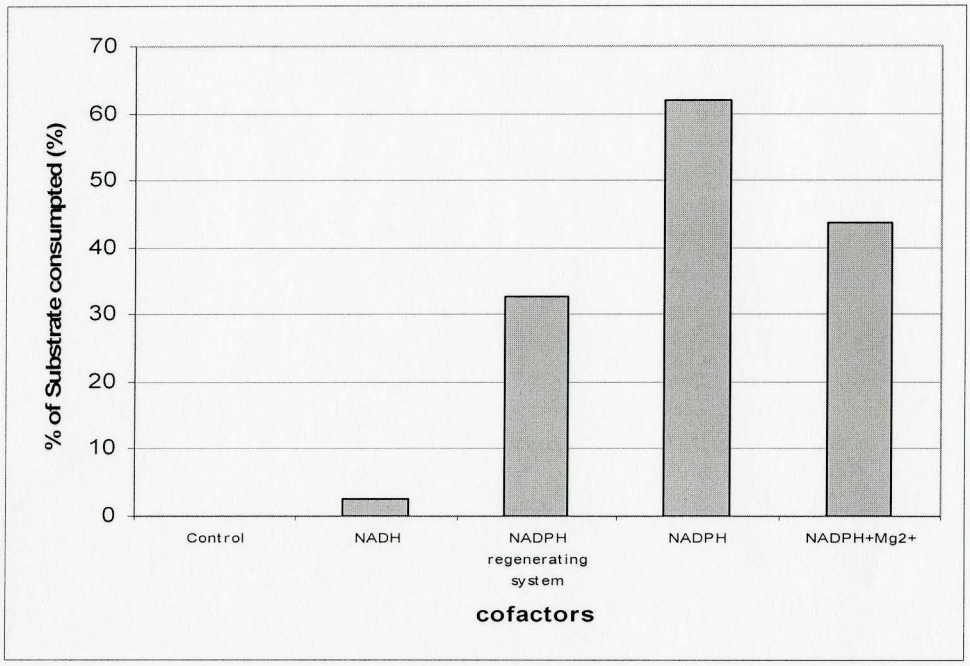


Figure 3-7. Effect of co-factors on the activity of TetX2. Control did not have any cofactor; NADPH regenerating system included $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase; Bars came from the average values of duplicate assays.

3.7 TetX2 requires molecular oxygen

Previous studies showed that TetX conferred tetracycline resistance in aerobically growing *E. coli* (Guiley et at., 1984; Speer et al., 1989). This implied a requirement for

molecular oxygen. To test this hypothesis, a reaction under non-O_2 conditions (N_2 saturation) was set up at room temperature for 20 minutes. HPLC analysis of the reaction progress showed a similar result as that of the negative control, which had no TetX2 added (Table 3-3). This result demonstrates that O_2 is a necessary factor for TetX2 function.

Table 3-3 The consumption of the substrate and the appearance of the product peak detected by HPLC followed reactions under O_2 or N_2 conditions

Reaction conditions	*% of substrate consumed (%)*	*Appearance of product peak*
Control (No TetX2)	0	No
Under oxygen condition	26	Yes
Non oxygen (N_2)*	0	No

* Non oxygen conditions were obtained by degassing all reaction components and incubating the reaction mixture in a plastic glove bag which was saturated with N_2.

3. 8 FAD dependence of TetX2 activity

TetX2 activity's dependence on FAD was monitored by measuring the reaction rate at the presence of varying FAD concentrations. The reaction rate increase with the FAD concentration from 0 to 2 μM; then, the rates remained unchanged regardless of the addition of increasing amounts of FAD (Fig 3-8).

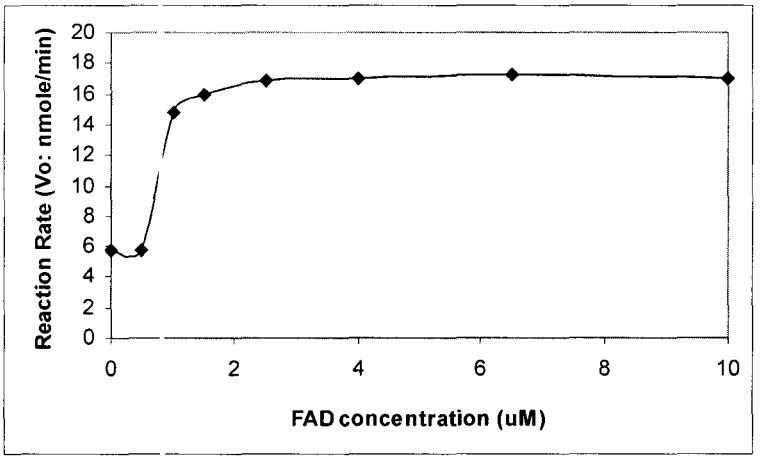


Figure 3-8. FAD dependence of the activity of TetX2. x axis is FAD concentration added; y-axis is the reaction rate.

3.9 TetX2 inactivates tetracyclines

The tetracycline inactivation activity of TetX2 was established by a series of biochemical assays. The difference between the products (inactivated tetracyclines) and the substrates (tetracyclines) of enzyme action can be detected by measuring the changes of the UV-Vis absorbance of the antibiotics. There are two absorption maxima in tetracyclines: 260 nm and 363 nm. The beta-tricarbonyl chromophore (ring A) is responsible for the 260 nm absorbance. The aryl beta-diketone chromophore (ring B, C, D) is responsible for the 363 nm absorbance and the yellow color of tetracyclines (Drexel et al., 1990). According to the observation of the disappearance of the yellow color when tetracycline incubated with TetX2, the continuous inactivation process should be determined by the absorbance change at 363 nm. Since NADPH has a maximum absorbance at 340 nm, the absorbance of both substrates overlap at 360 nm. Therefore,

the absorbance at 400 nm (ε_{400} of oxytetracycline is 1080 M^{-1} cm^{-1}) was chosen when the plate reader was used. Thus the activity of TetX2 was detected when the progress curve was observed with a negative slope, indication of tetracycline inactivation.

3.9.1. UV-Vis detection of the activity of TetX2

a. *Difference spectra:* The difference spectra of oxytetracycline between incubation with TetX2 and without TetX2 during 20 minutes were recorded by UV-Vis spectrophotometer. In the sample cuvette (+TetX2), the absorbance of oxytetracycline at 363 nm decreased while in the reference cuvette, the absorbance maintained stable.

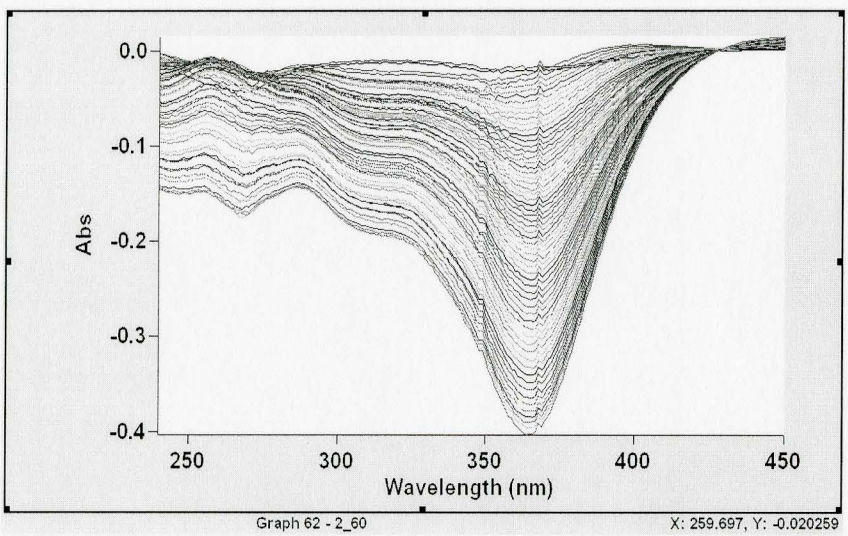


Figure 3-9. Difference spectra of oxytetracycline when incubated with TetX2 for 20 minutes. The top flat line is recorded at 0 minute, the bottom curve is recorded at 20 minutes. Abs. is the difference between the sample and the reference.

b. *Absolute absorbance spectra*: The absorbance spectra of oxytetracycline when incubated with TetX2 is shown in Fig. 3-10. The decreasing of oxytetracycline

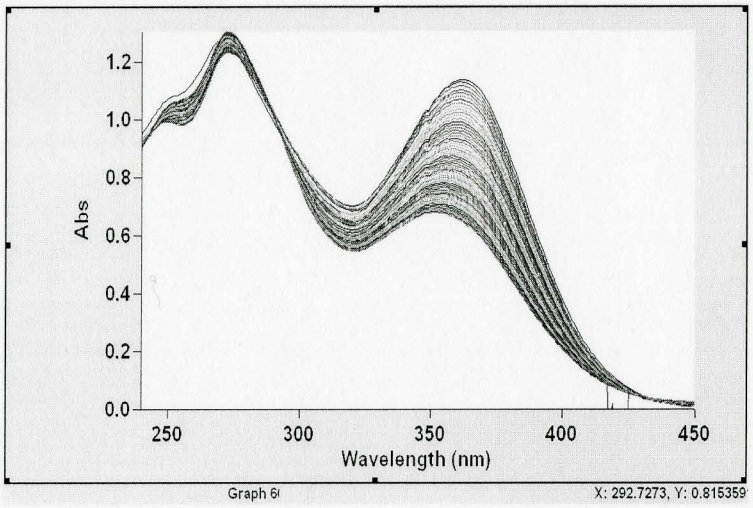


Figure 3-10. Absolute absorbance spectra of oxytetracycline when incubated with TetX2 for 20 minutes. The curves from top to bottom are recorded from 0 minute to 20 minutes. The decreasing of absorbance at 363 nm very obvious with the increasing of incubation time.

absorbance at 363 nm is very obvious with the increasing of incubation time during a 20-minutes time course (Fig. 3-10). The absence of the isosbestic points indicated a more complex transition form substrate to product.

3.9.2 HPLC detection of the activity of TetX2

The disappearance of the substrate peak and the appearance of product peaks were detected by reverse phase HPLC when oxytetracycline was incubated with TetX2. Figure

3-11 showed the changes during a 30-minutes time course. With the increase of the reaction time, the substrate peak (S) decreased and a product peak (P1) increased. It was noticed that the first product (P1, RT: 11.3 minutes) seems to be an intermediate. It appeared at 2 minutes and reached the highest at 15 minutes, then started to decrease. Product 2 (P2) appeared at 10 minutes and kept increasing until the reaction was stopped (30 minutes).

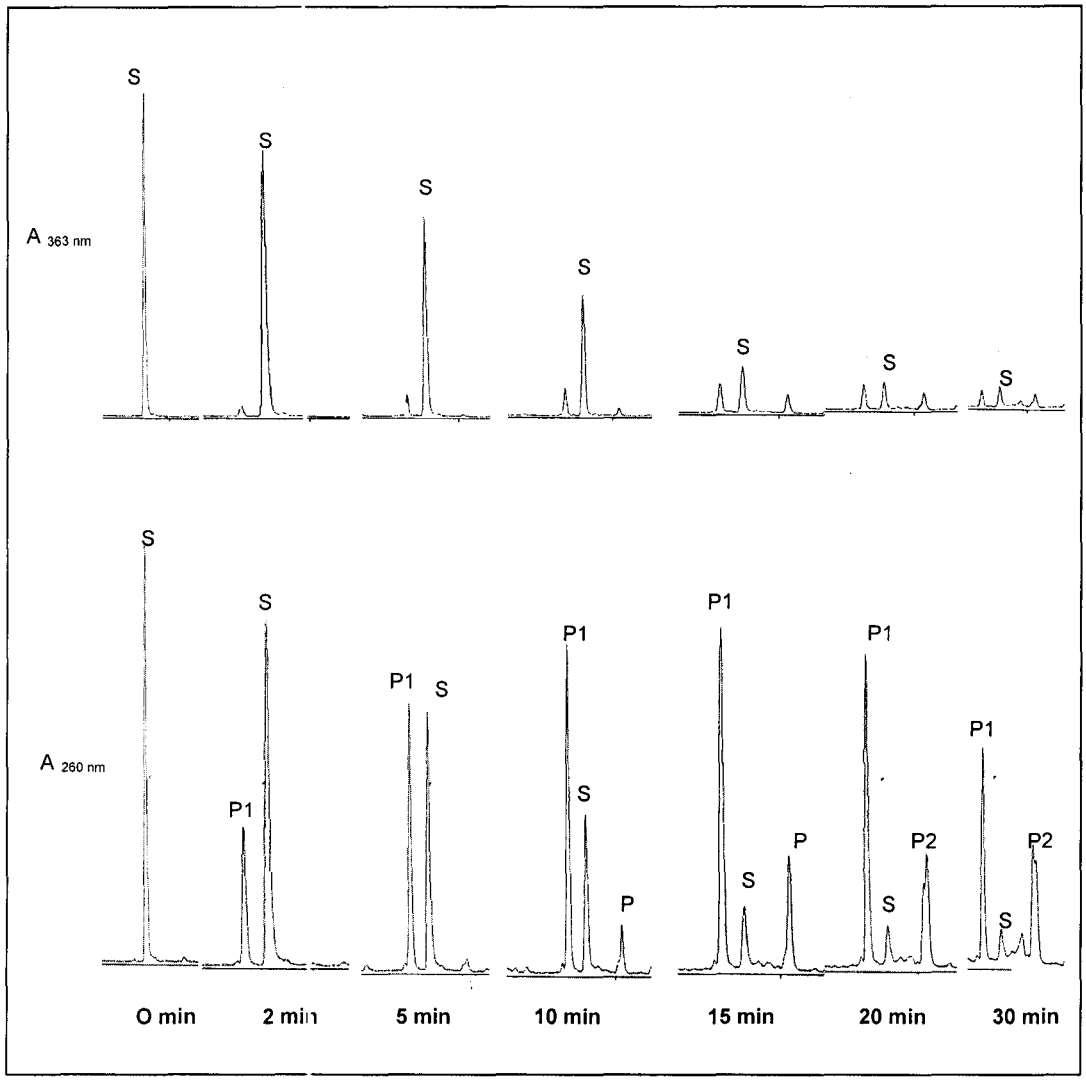


Figure 3-11a. HPLC detection of the substrate consumption and the product formation by TetX2. Indicated are samples of oxytetracycline incubated with TetX2 for 0 minute to 30 minutes. S-substrate, oxytetracycline; P1-1st product; P2-2nd product. The top peaks were detected at 363 nm, the bottom peaks were detected at 260 nm.

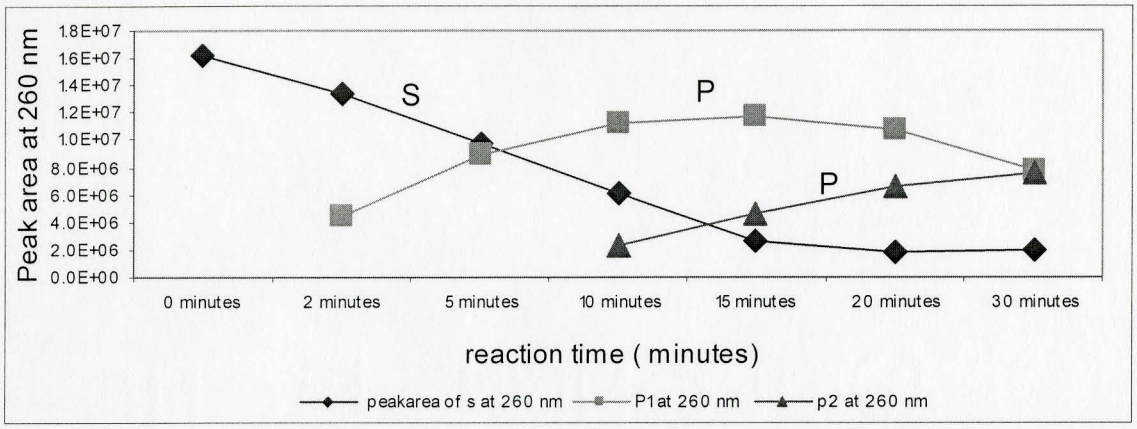


Figure 3-11b. Quantification of HPLC data. From 0 to 30 minutes, substrate is decreasing; the first product (P1) appears at 2 minutes, increases up to 15 minutes; the second product (P2) appears at 10 minutes, and it keeps increasing.

3.9.3 Effect of TetX2 on antibiotic activity of oxytetracycline

A microbiological disc assay showed the change in bacterial antibiotic susceptibility to oxytetracycline in the reaction mixture. A tetracycline sensitive strain, *Micrococcus luteus,* was used (Fig.3-12). With the increasing of the reaction time, the amount of active oxytetracycline in the reaction mixture decreased due to the inactivation catalyzed by TetX2.

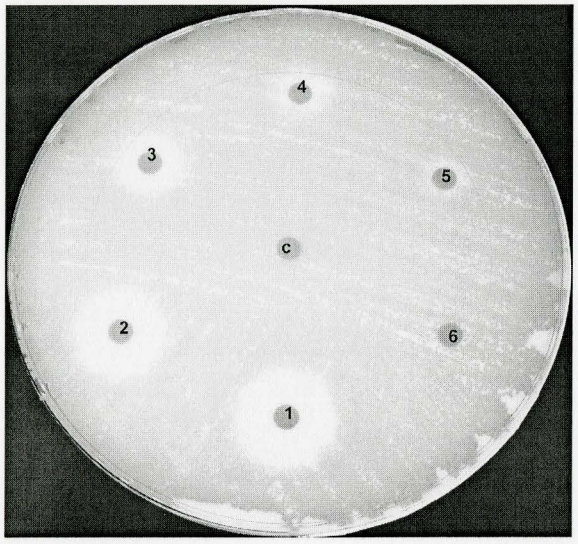


Figure 3-12. Detection of bacterial susceptibility to inactivated tetracycline by disc assay. A tetracycline sensitive strain *Micrococcus luteus* was used. c: control, without oxytetracycline; 1 to 6 are samples with 30 μmole oxytetracycline incubated with TetX2 for 0 minute, 5 minutes, 10 minutes, 15 minutes, 20 minutes, and 30 minutes.

3. 9.4. Steady state kinetic data of TetX2 catalyzed tetracycline inactivation

In tetracycline inactivation reaction, TetX2 uses both tetracycline and NADPH as substrates. Therefore, steady state kinetic data of TetX2 were collected separately by varying oxytetracycline concentrations and NADPH concentrations in separate experiments.

Fig. 3-13a shows the result of varying oxytetracycline concentrations. The calculated k_{cat} is 1.32 s^{-1}, k_{cat} /K_m is 1.74 x 10^3 $M^{-1}.s^{-1}$, and K_m is 76.3 ± 16.3 μM. Fig. 3-13b shows the result of varying NADPH concentrations. The calculated k_{cat} is 1.11 s^{-1}, k_{cat} /K_m is 8.32 x 10^3 $M^{-1}s^{-1}$, and K_m is 133 ± 25.6 μM.

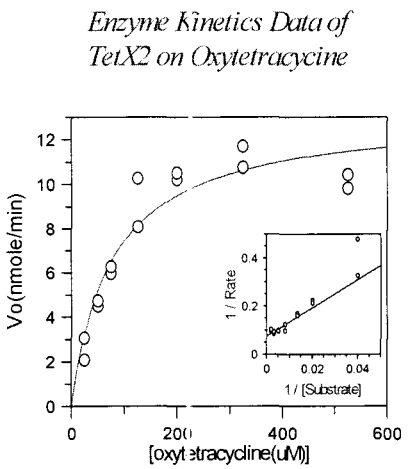


Figure 3-13a Steady state kinetics of TetX2. Varying concentrations of oxytetracycline, 1 mM NADPH and 25 mM TAPS buffer, pH 8.5, were used.

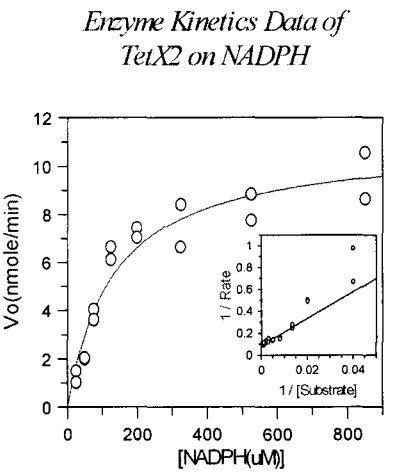


Figure 3-13b Steady state Kinetics of TetX2. Varying concetrations of NADPH, 0.3 mM oxytetracycline 25 mM TAPS buffer, pH 8.5, were used.

The kinetic data of TetX2 on several other analogues of tetracyclines were also tested to compare the substrate specificity of the enzyme. The results shown in table 3-3 indicates that TetX2 can catalyze the inactivation reactions of the chosen analogues of tetracyclines, even though the reaction rates are different.

Table 3-4 Steady state kinetic data of different tetracycline analogues

Substrate	Km(μM)	k_{cat} (s^{-1})	k_{cat}/Km ($M^{-1}.S^{-1}$)
Oxytetracycline	76.3 ± 16.3	1.3 ± 0.07	1.74×10^4
Demecolocycline	19.9 ± 7.10	0.2 ± 0.01	1×10^4
Doxycycline	83.7 ± 27.2	0.63 ± 0.09	7.52×10^3
Tetracycline	54.0 ±11.5	0.32 ± 0.02	5.92×10^3
Minocycline	28.4 ± 8.8	0.12 ± 0.01	4.09×10^3
Chlortetracycline	110 ± 12	0.3 ± 0.01	2.74×10^3

TetX2 has activity with all of them, but the reaction rates are different. 1 mM of NADPH and 25 mM of TAPS buffer, pH 8.5, were used in the reactions.

3. 9. 5. Time course analysis of selected substrates

Analysis of the formation of products of tetracyclines by TetX2 action was also explored with tigecycline and chlortetracyline (Fig. 3-14 and 3-15).

Fig. 3-14a shows the changes of the substrate and products peaks during the inactivation catalyzed by TetX2 when tigecycline was used as the substrate. After 20 minutes reaction, no substrate peak was detected. Fig. 3-14b is the quantitative graph of the HPLC data. The structure analysis of P was not investigated due to the limited supply of tigecycline. The decreasing of substrate and the increasing of one product was shown clearly. Fig. 3-14c shows the antibiotic activity of inactivated tigecycline by disc assay. Corresponding to HPLC result, after a 120 minute reaction, the reaction mixture was not toxic to bacteria at all due to the completion of the inactivation of tigecycline catalyzed by TetX2.

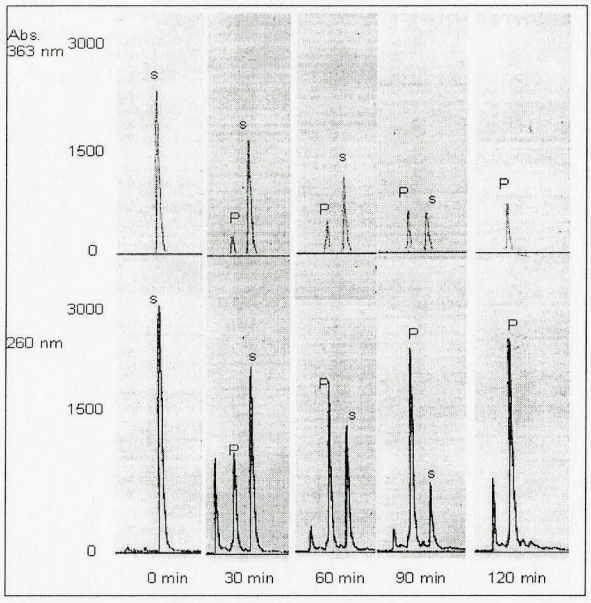


Figure 3-14a HPLC detection of tigecycline inactivation catalyzed by TetX2. S: substrate peak; P: product peak. X-axis is the reaction time, Y-axis is the absorbance. The top peaks were recorded at 363 nm; the bottom peaks were recorded at 260 nm.

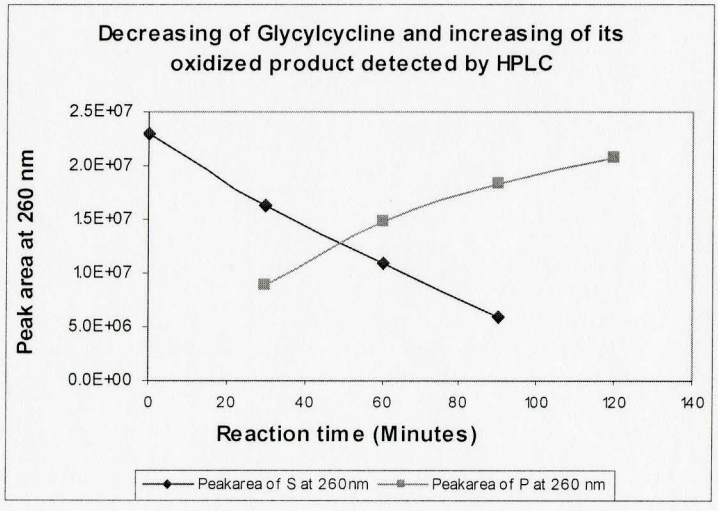


Figure 3-14b Quantification of the HPLC data of 3-14a. X-axis is the reaction time, Y-axis is the peak area at 260 nm.

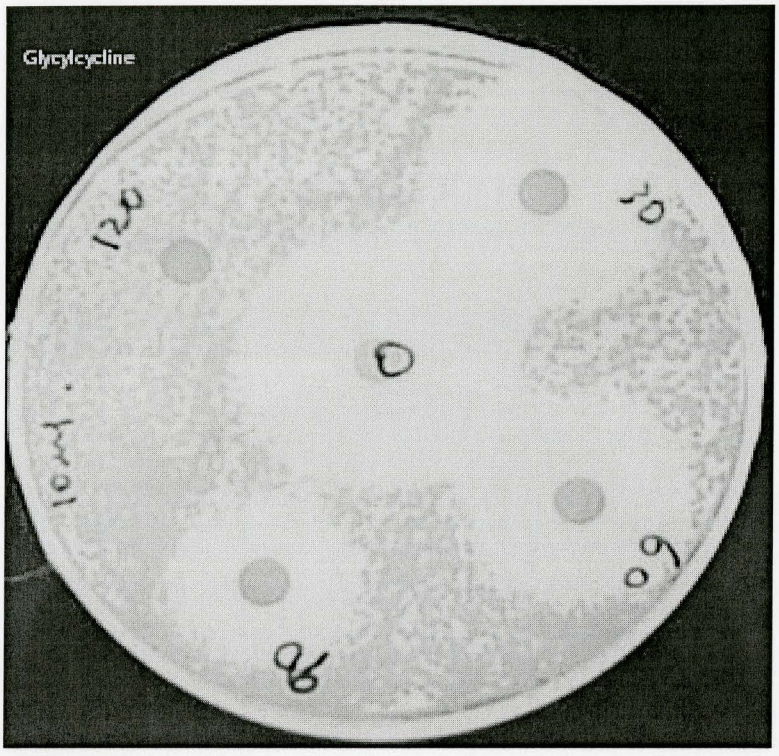


Figure 3-14c. Disc assay for the detection of the inactivation of tigecycline by TetX2. A tetracycline sensitive strain *Micrococcus luteus* was used. Numbers are the incubation time before the reactions were stopped by adding 1 % TFA, the units are minutes.

Fig.3-15a and b are corresponding results of the HPLC trace, quantification of HPLC data when chlortetracycline was used as the substrate. HPLC result showed the disappearance of the substrate and the appearance of 3 new products when chlortetracycline was incubated with TetX2. We did not pursue the characterization of these peaks further due to time constrains. The decreased antibiotic activity of inactivated chlortetracycline by TetX2 was shown by micro disc. assay (Fig. 3-15c).

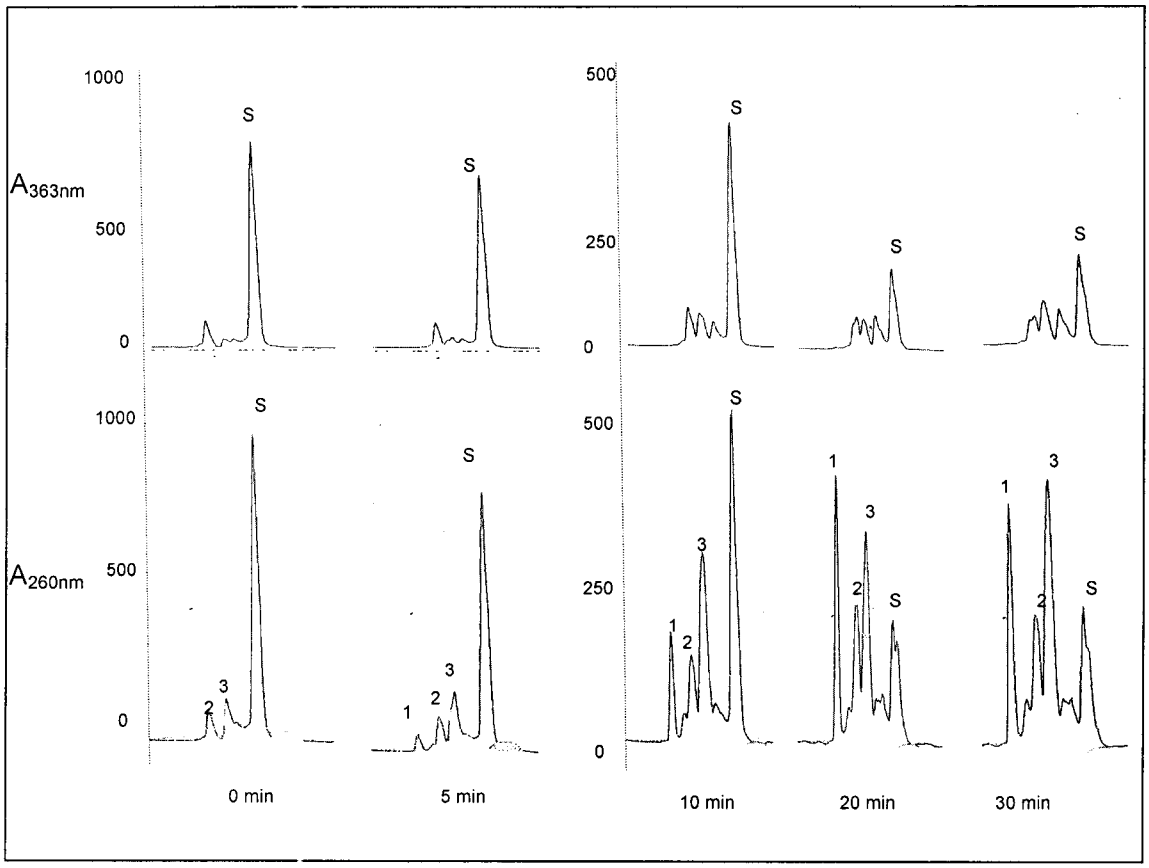


Figure 3-15a HPLC detection of the changes in tetracycline inactivation catalyzed by TetX2. The substrate is chlortetracycline, represented by number 4. Peak 1, 2, and 3 are possibly the inactivated products. X-axis is the reaction time. Y-axis is the absorbance, the scale for 0 minute and 5 minutes is different from the scale for 10 minutes to 30 minutes due to the printouts.

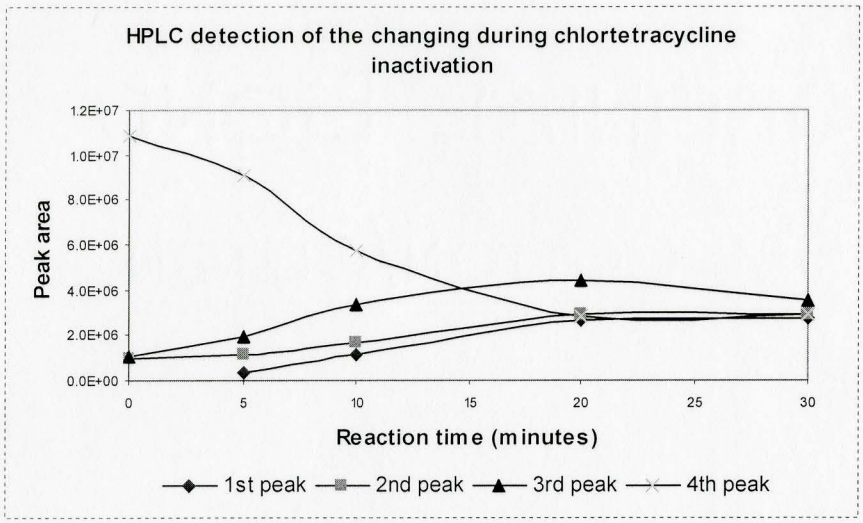


Figure 3-15b Quantification of HPLC data. Data used were peak area recorded at 260 nm. 1st, 2nd, and 3rd peaks are products, 4th peak is the substrate.

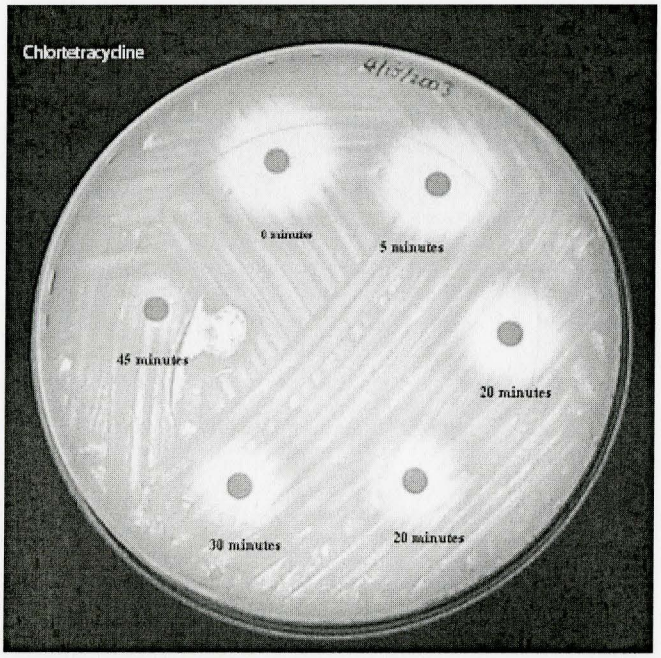


Figure 3-15c Micro disc assay showed the decreased antibiotic activity of inactivated chlortetracycline. Samples were chlortetracycline incubated with TetX2 for different time periods.

3.9.4 Resistance of *tetx2* carrying *E. coli* to tetracyclines

The resistance profile of *tetx2* carrying *E. coli* was performed by testing the minimum inhibition concentrations (MICs) of tetracyclines (Table 3-5). When bacteria were carrying pET28-*tetx2*, it conferred resistance to some tetracyclines, but not minocycline and tigecycline. However, the MICs are lower than bacteria carrying pBR322, which has a *tetC* gene encoding an efflux protein.

Table 3-5 Minimum inhibition concentrations (MICs) of tetracyclines to *E.coli* BL21-*tetx2* (μg/ml)

Strains	Oxytetracycline	Tetracycline	Chlortetracycline	Doxycycline	Minocycline	Tigecylcycline
pET28	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
tetx2	8	2	4	1	<0.5	<0.5
pBR322	128	16	8	2	1	0.5

3.10 Identification of product of TetX2 catalyzed inactivation of oxytetracycline

3.10.1 LC- MS spectra of P1, P2.

The molecular masses of P1, P2 were determined by LC-MS at the department of Chemistry, University of McMaster. Figure 3-16 shows that the first product of inactivated oxytetracycline (P1) has a mass of 477 Dalton, which is equivalent to oxytetracycline (461 Da) with one oxygen (16 Da) added, implying a mono-oxidation process occurred. The mass of the second product (P2) is 457 Dalton.

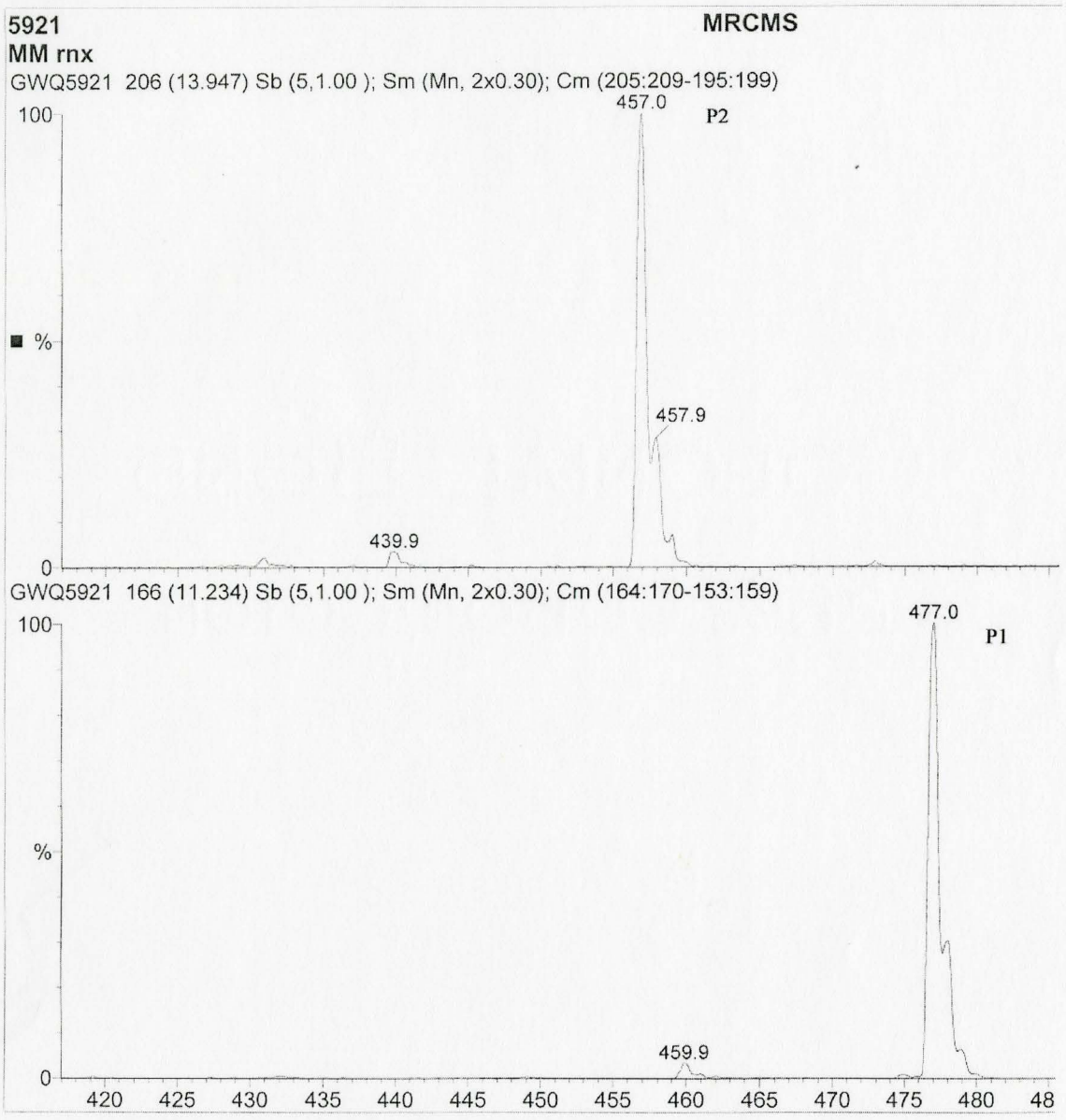


Figure 3-16 LC-MS of oxidized oxytetracyline products. Positive ion electron spray was performed. The top peak is P2, the bottom peak is P1.

3.10.2 NMR spectrum of P1

From mass spectrum, the structure of the product cannot be determined. In other words, the oxidation position of oxytetracycline was not yet clear. NMR has been a powerful tool for determining the structure of compounds, so we decided to take

advantage of this technique, in collaboration with Dr. D. Hughes, Department of Chemistry of McMaster University.

a. *Optimizing the reaction time to obtain maximum P1* To obtain the maximum yield of the product, we optimized the reaction time. Incubating 300 μM oxytetracycline with 10 μg TetX2 at 30°C for 18 minutes converted most of the substrate into products (Fig. 3-17). The product P1 separated from that reaction was one single peak shown on analytical HPLC (fig. 3-18).

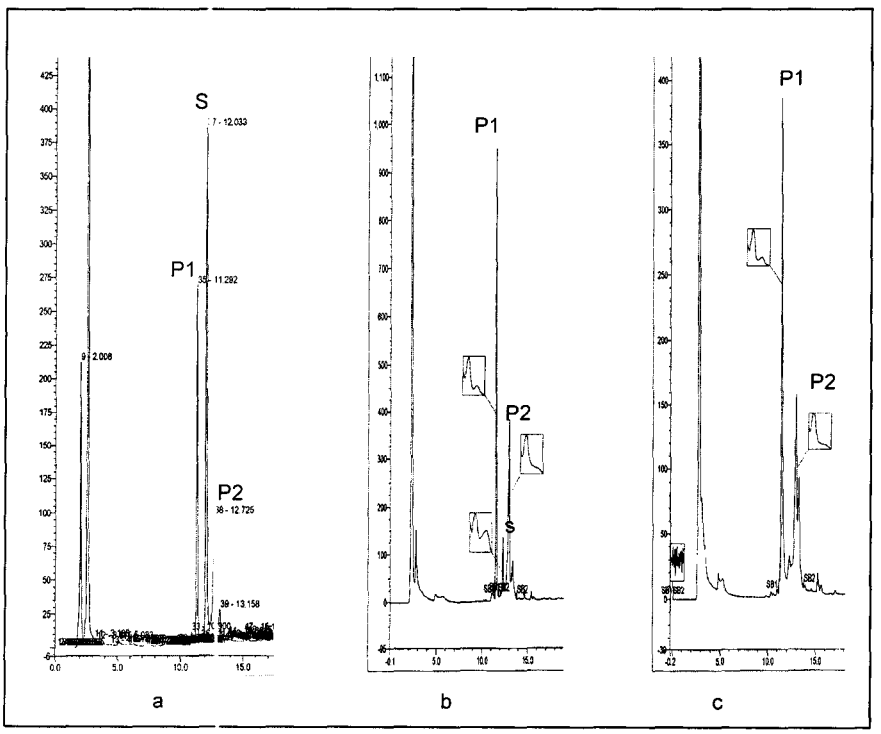


Figure 3-17. Different reaction times give different amount of products. Indicated are different reaction times: a,10 minutes; b, 15 minutes; c,18 minutes. Numbers on the peaks are Retention time of corresponding peaks; The boxes are the spectra (220 to 450 nm) of relevant peaks.

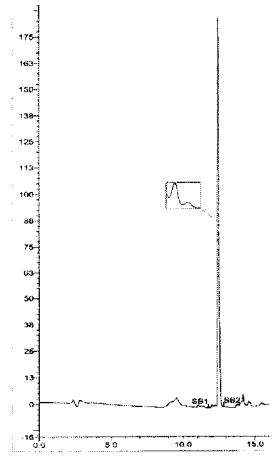


Figure 3-18. Purified P1 was verified by analytical HPLC. One single peak was shown with the right spectrum.

b. NMR spectra of P1 The ^{1}H NMR spectra of the inactivated product P1 was shown in figure 3-19. Table 3-6 showed assignments for the protons and based on this data, the structure was proposed (Fig 3-20). The oxidation was occurred at the 5a position of the B-ring of the oxytetracycline.

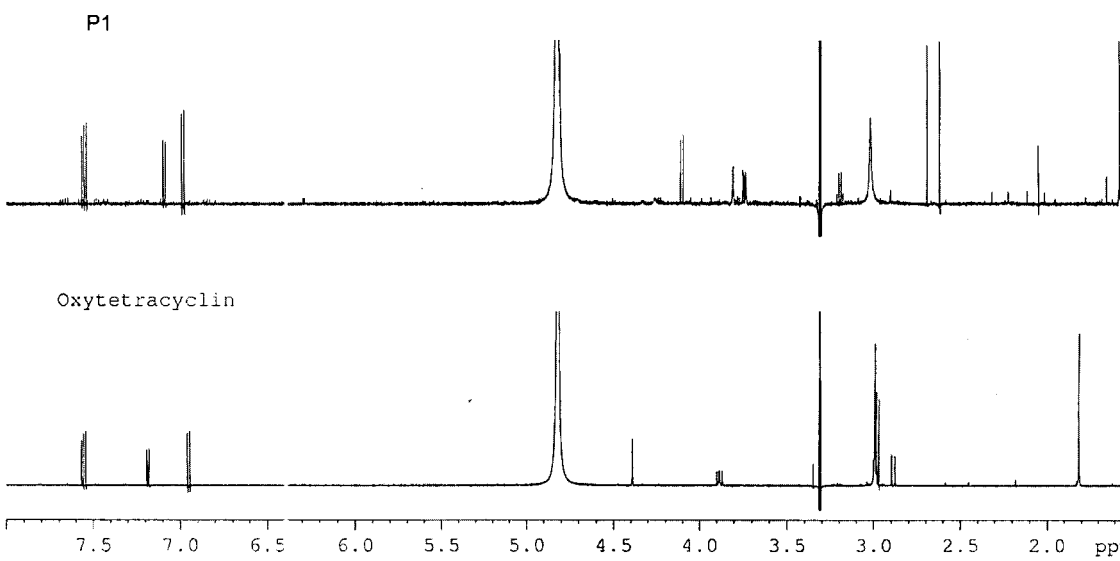


Figure 3-19. ^{1}H NMR spectra of oxytetracycline and its inactivated product, P1.

Table 3-6 Assignments of protons of oxytetracycline and its inactivated product P1 from ^{1}H NMR

proton	*4*	*4-$(NH_3)_2$*	*4a*	*5*	*5a*	*6-CH_3*	*7*	*8*	*9*
Oxytetracycline	4.389	2.990	2.885	3.882	2.974	1.814	7.184	7.554	6.951
P1 (Aug.27)	3.803	3.019	3.740	4.101		1.576	7.090	7.551	6.984
P1 (Sep. 3)	3.515	2.907	3.557	4.116		1.595	7.079	7.537	6.966

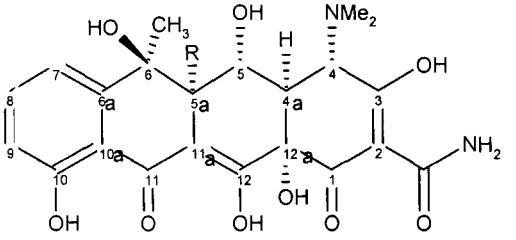


Figure 3-20. Proposed structure of P1. R: H for oxytetracycline, OH for P1.

3.10.3. *LC-MS-MS:* LC-MS-MS of the products was conducted and the fragments (Table 3-7) matched the proposed structure of P1. The structure of P2 is not clear because we couldn't obtain the pure compound due to the instability.

Table 3-7 Molecular mass and proposed structures of the precursors and product ions

Precursor Ion (m/z)	Oxytetracycline (461)	P1(478)	P2 (457)	Proposed structures$[M+H]^+$
Most abundant product Ion (m/z)		460		$[M+H-H_2O]^+$
	444	444		$[M+H-2xH_2O]^+$
			440	$[M+H-NH_3]^+$
	426			$[M+H-NH_3-H_2O]^+$
	408	406		$[M+H-NH_3-2xH_2O]^+$
	381	379		$[M+H-NH_3-2xH_2O-CO]^+$
	337	336	333	$[M+H-NH_3-2xH_2O-CO-N(CH_3)_2]^+$

3.11 **P1 to P2 reaction:**

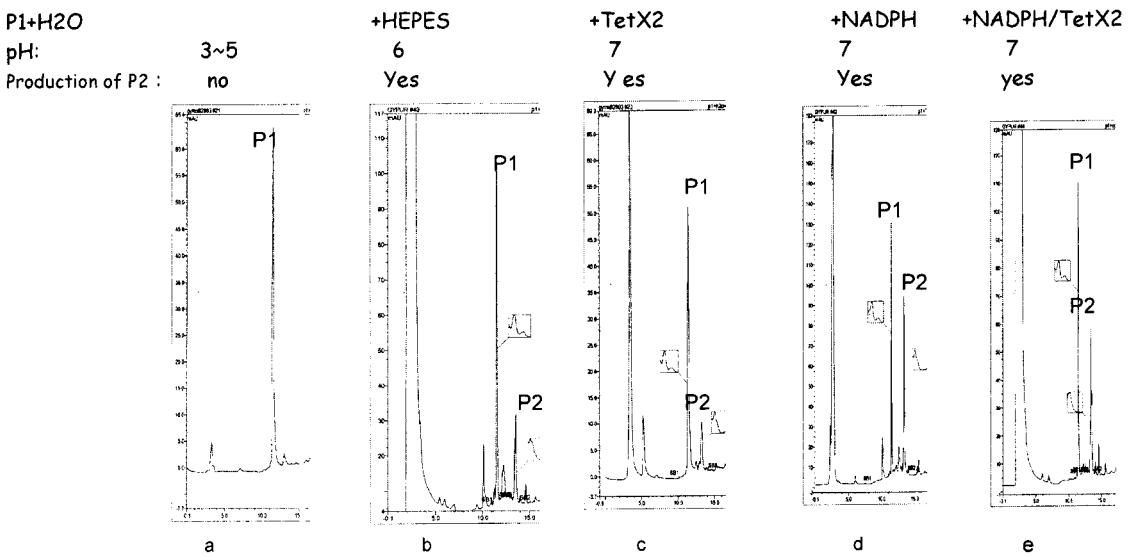


Figure 3-21. HPLC chromatographs showing the production of P2 when using P1 as the substrate. a to e are reactions with different pH value and whether TetX2 was included. Figures in the boxes are the spectra of corresponding peaks.

HPLC separated P1 was used as the substrate and reaction was set up as the same as for enzyme activity. Two controls were used: one was P1 in H_2O (a); the other was P1 in H_2O with 2 mM HEPES (pH 8.0) (b). To compare the difference of the reaction with TetX2 and the reaction without TetX2, three samples were prepared as follows: c), P1 incubated with TetX2; d), P1 plus TetX2 and NADPH regeneration system; e), P1 plus NADPH regenerate system, without TetX2. The pH of these three samples and the two controls were taken and they were incubated at room temperature for 20 minutes, then the reactions were stopped by adding 1% TFA and the changing of the substances were detected by RP-HPLC.

Fig 3-20 shows that TetX2 was not required for the transfer of P1 to P2. This process is therefore a pH-dependent P1 auto-degradation, not an enzymatic process.

Chapter 4

Conclusions and discussions

The objectives of this work were to study the molecular aspects of tetracycline inactivation by TetX2. To accomplish this goal, a series of biochemical assays were established. They have been used successfully to study the biochemical properties of TetX2 and it's catalyzed tetracycline inactivation process. Previous investigators had tried different assays to study TetX. Since they did not isolate the protein from bacterial cells, most of the results were indirect, often confusing. For example, HPLC and spectrophotometric assays had been used to fellow tetracycline inactivation, it was pointed out that the effect of cell secretion factors couldn't be eliminated (Speer et al., 1989; 1991). For example, a weak efflux pump was shown to be involved and it was suggested that the protein product of *tetx* gene might have both tetracycline inactivating activity as well as tetracycline efflux function (Park et al., 1988). It is likely that this confusing assumption was based on experiments with the bacterial spent media that included other interfering factors and cell secretion factors. That might be one of the reasons why no further studies had been reported after 1991. Using purified hexa-His TetX2, we were able to monitor the tetracycline inactivation activity of the enzyme directly, and in particular the consumption of tetracycline was monitored by spectrophotometer and HPLC chromatography.

Characterization of the size of TetX2 by SDS-PAGE electrophoresis and gel filtration chromatography showed that TetX2 is 44 kDa, which was in agreement with the predicted size by sequence analysis (Speer and Salyers,1989). However, previous results obtained from maxicel experiments didn't prove conclusively that the 44 kDa

cytoplasmic protein shown on SDS-PAGE was the *tetx* gene product (Speer et al., 1988). Our result clearly described that TetX2 is the protein product of gene *tetx2* and is about 44 kDa.

TetX2 was purified with a bound flavin cofactor FAD. Though a flavin binding sequence was not identified in the original TetX deduced sequence by Speer and her colleagues (Speer and Salyers, 1989), a GGGPVG sequence in TetX2 protein matches the first fingerprint motif of GXGXXG for the FAD binding (Eppink et al., 1997). A blast search found 10 hits (Fig.4-1) with the E value less than 1.0. Nearly all of them encoded hydroxylases/mono-oxygenases.

```
                *         20          *         40          *         6
TetX2 : --------------MTMRIDTDKQMNLLSDKNVAIIGGGPVGLTMAKLLQQNGIDVSVY :  45
2     : --------------MTMRIDTDKQMNLLSDKNVAIIGGGPVGLTMAKLLQQNGIDVSVY :  45
3     : ------------------------MTLLKYKKITIIGAGPVGLTMARLLQQNGVDITVY :  35
4     : ----------------------MEQSAINTKKIAIVGGGPGGLTLARLLQKKGADVHVY :  37
5     : ---------------------------MNHVPVAIIGAGPAGLTLAHLLHLQGIESVVF :  32
9     : ---------------------------MNHVPVAIIGAGPAGLTLAHLLHLQGVESIVF :  32
8     : -----------------------------MKTQVAIIGAGPAGLLLGQLLHKAGIDTVIL :  31
6     : MTIEFTSYPFTAQHYSARLPSLGGGVETSRHAVAIVGGGPVGLTLALGLAKHGIRCVLI :  59
10    : ---------------MAIQHPDIQPAVNHSVQVAIAGAGPVGLMMANYLGQMGIDVLVV :  44
7     : -------------------MHYEELPLTAEHDVAIVGAGPIGLEVAVCLKQAGVDYIQF :  40
                                    6aI G GP GL 6a  L   G
```

Figure 4-1. An ADP-binding fingerprint (GXGXXG) for FAD is present in TetX2. 2, Original TetX; 3, A tetracycline inactivating enzyme from *Pseudomonas aeruginosa*; 4, 2-polyprenyl-6-methoxyphenol; 5, Hydroxybenzoate3-monooxygenase; 6, 2-polyprenyl-6-methoxyphenol hydroxylase; 7, hypothetical protein; 8, p-hydroxybenzoate hydroxylase [*Pseudomonas* spp.]; 9, 2-polyprenyl-6-methoxyphenol hydroxylase [*Corynebacterium glutamicium*]; 10, 3-(3-hydroxyphenyl) propionate hydroxylase [*E.coli*].

The presence of flavin in TetX2 suggested either a redox reaction or a monooxygenase action. Assays in the presence and absence of oxygen reveled that O_2 is

required for TetX2 activity, which clearly explained why *E.coli* carrying *tetx* gene only grow aerobically in the presence of antibiotic (Guiney et al., 1984; Speer and Salyer, 1989). Characterization of the requirements for the TetX2 function showed that the nicotinamide co-substrate NADPH is exclusively required for activity. Steady state kinetic data of TetX2 obtained using oxytetraycline and NADPH separately indicated that TetX2 used both substrates. These experiments classified TetX2 is an O_2 requiring falvin monooxygenase.

TetX2 demonstrated a broad substrate specificity on tetracyclines. But it also exhibited differences in the reaction rates among these antibiotics. The *in vitro* k_{cat} /Km value were in the 10^{3}- 10^{4} M^{-1}. s^{-1} range. These observations were in agreement with Speer and Salyer's microbiological assay results, which showed an *E. coli* strain, EM 24, carrying *tetx* gene conferred tetracycline resistance to several tetracyclines derivatives with the MICs value range of 20 to > 200 μg / ml (Speer et al., 1989).

From the structure-activity point of view, the variations of the analogues we have used are at the C5, C6, C7 positions. That means these positions are not involved in the inactivation process. It is supported by the proposed structure of oxidized oxytetracycline product, where the substrate is oxidized at C-5a position.

Microbiological assay showed that *E.coli* strain BL21(DE3) transformed with *tetx2* conferred tetracycline resistance, even though the ability of the resistance was not very strong. Speer and Salyers (Speer et al., 198 reported a much higher resistance (MICs

>200 μg/ml) when strain EM24 was used (Speer and Salyers, 1988). Park and colleagues reported a relatively lower resistance (MICs <25μg/ml) when strains JF270 and HB101 were used (Part et al., 1988). The differences between experiments suggest the performance of *tetx2* was likely affected by different strains.

An interesting phenomenon was noticed in that there was no correlation between MIC of individual antibiotics and the steady state enzyme reaction rates k_{cat} or k_{cat} / K_m (Fig 4- 2). A positive correlation between MIC and k_{cat} / K_m is expected for an efficient resistance enzyme (Wright and Ladak, 1997). One possible explanation is that the primary function of TetX2 is not to confer tetracycline resistance. It might be involved in a complex bacterial metabolic pathway when it is *in vivo*. Further studies will provide a better understanding of the function of TetX2 both *in vivo* and *in vitro*.

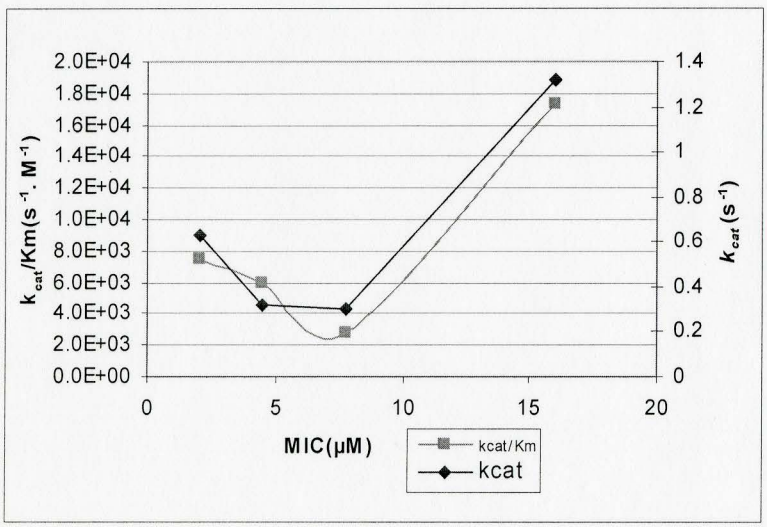


Figure 4-2 The relationship between MICs and the enzyme reaction rates. No correlation was shown in the graph.

Initial attempts to determine the molecular weight and the chemical structure by mass-spectrometry and NMR failed due to the existence of interfering materials (Speer

and Salyers, 1989). Using purified TetX2 and modified product separation techniques, we were able to overcome the obstacles caused by the instability of the product and gained pure material for mass-spectrometry and NMR. The molecular mass of inactivated oxytetracycline product catalyzed by TetX2 was 477 Daltons, which is equivalent to one oxygen atom added to the molecule. NMR analysis of the product P1 demostrated that the oxidation was a hydroxylation of the C_{5a} position of oxytetracycline.

Characterization of the biochemical mechanism of the inactivation process indicated that one oxygen atom is incorporated into tetracycline during tetracycline inactivation. Thus TetX2 is a monooxygenase. This is consistent with the classfication made by V. Massey: the fourth group of flavoprotein-monooxygeneases induces a splitting of the O-O bond, inserting one oxygen atom into a substrate and reducing the other atom to H_2O (Massey, 1994). He also generalized that all true monooxygenases have three substrates, NAD(P)H to reduce the enzyme bound flavin, the substrate to be oxygenated, and molecular oxygen (Massy, 1994). Therefore our experimental results could be used to enrich the supportive evidence of biochemical theories.

Assay for the fate of tetracycline inactivated product (P1) indicated that the oxidized product was not stable, but is easily degraded by increasing the pH of its environment. P2 is likely not a single compound, but several. In the process of cultivation of *tetx2* carrying bacteria, we have notice the formation of the dark color in the cultural media, which is likely an indication of the breakdown of the inactivated product P1. Due to the diversity, we were not able to generate products P2 and

characterize them. However, the molecular weight of the major degraded product is 457 Dalton by LC-MS. To understand more about the fate of oxytetracycline inactivated product, further studies of P2 are required.

According to *tetx2* sequence, there are 14 potential start codons (Met) in the 1.2 kb fragment. By base deletion experiments, it was found that putative start codons at position 1, 3, or 11 could be the actual start codons (Speer and Saylers, 1991). We have cloned two versions of *tetx2* which start from position 1, and 11. The proteins from them were purified and the activities for tetracycline inactivations were tested. Both versions of TetX2 showed tetracycline inactivation activities. A version of TetX with one mutation (E266K) (TetX3) showed tetracycline inactivation activity as well. The *tetx2* gene is carried by transposon, a mobile genetic element. If the horizontal gene transfer is involved, the spread of enzymatic tetracycline inactivation type of resistance determinants has the potential to be very fast. The fact is the enzymatic inactivation of antibiotics is a common mechanism in other antibiotics (eg. β-lactams, aminoglycosides). For *tetx2,* the clinical relevance has not yet been reported. Its original host, *Bacteroides,* has been reported as major carrier of resistance gene transfer (Shoemaker et al., 2001). And a gene, *tet37*, encodes the same general mechanism as *tetx2*, but there is no sequence homology, was reported (Torries et al., 2003). Therefore, further studies and surveys about the distribution of *tetx* type tetracycline resistance determinants are significant.

Our attempts to characterize the 3D structure of TetX2 have thus far not been successful. Further studies have to be done to overcome the obstacles for crystallization

of TetX2. The results would clearly shed light on the understanding of the enzymatic tetracycline inactivation mechanism.

Extensively using tetracyclines, especially in agriculture, not only make the resistance problem more serious, but also cause environmental problems which are associated with the decomposition of feed-lot wastes containing tetracyclines. The wastes resulting from animals treated with antimicrobials, regardless of whether the animals were managed in a feed-lot operation or on farms, will find their way to the agricultural field (Halling-Sørensen et al., 2002). Our work on TetX2 suggests that large scale production of this enzyme is possible. It could therefore be used as a tetracycline detoxifying agent to treat tetracycline contaminated waste or agricultural field, solve environmental problems.

To summarize: the *tetx2* gene has been cloned, the protein was overexpressed in *E.coli* and purified by column chromatography; the size of TetX2 protein is 44 kDa on SDS-PAGE and confirmed by gel filtration; TetX2 was characterized as a flavoprotein bound with FAD; TetX2 exhibited enzymatic activity on tetracycline inactivation, and this inactivation required oxygen and NADPH; the product of TetX2 inactivated oxytetracycline was an oxidized compound with a mono oxygen atom added on the C-5a position of the substrate with a molecular weight of 477 Dalton; thus the biochemical process of tetracycline inactivation is a TetX2 catalyzed oxidation reaction; the oxidized product was not stable, it will degrade when the pH of its environment rise up to 6.

It is expected that these results will open up a new view for the better understanding of tetracycline resistance mechanism, especially enzymatic tetracycline inactivation mechanism. Such Results could be helpful in the battle of fighting with tetracycline resistance problem and bring the broad-spectrum antibiotic agents, tetracyclines, back to the forefront of modern antimicrobial chemotherapy.

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